Journal of Clinical Toxicology
ISSN: 2161-0495

Shehata et al., J Clin Toxicol 2013, 3:4
DOI: 10.4172/2161-0495.1000169

Research Article Open Access

Effects of Exposure to Plasticizers Di-(2-Ethylhexyl) Phthalate and Trioctyltrimellitate on the Histological Structure of Adult Male Albino Rats' Liver

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Abstract

Di (2-ethylhexyl) phthalate (DEHP) and Trioctyltrimellitate (TOTM) are plasticizers used to increase the flexibility of polyvinyl chloride. Their wide commercial usage carries high risk of human exposure. This work aimed to investigate histological changes of rat's liver upon DEHP and TOTM exposure. 36 adult male albino rats were classified into 3 equal groups; control group, group II (given DEHP 300 mg/kg) and Group III (given TOTM 300 mg/kg) orally for 4 weeks. Half of the animals were sacrificed after one day of last dose (subgroups IIA & IIIA) and the remaining were left for another 4 weeks for recovery (subgroups IIB & IIIB). Liver specimens were examined by light and electron microscopes and immunohistochemical stain for hepatocyte paraffin -1 (Hep Par-1). By examination of subgroup IIA all rats' liver showed focal changes. Dramatically affected lobules revealed loss of lobular architecture with periportal cellular and fatty infiltrations. Hepatocytes had shrunken nuclei, peroxisomes and lipid globules. Subgroup IIB showed mild morphological improvement. Subgroup IIIA showed preserved lobular architecture with vascular congestion. Hepatocytes showed euchromatic nuclei and lipid globules. Subgroup IIIB revealed apparent normal lobular architecture and normal hepatocytes. Comparing to control group, the area % of Hep Par-1 immune reaction in subgroup IIA was highly significant decrease while group IIB was significantly decreased. Subgroup IIIA was significantly decreased. However, group IIIB had no significant difference with control. In conclusion, DEHP induced serious changes on liver; most of these changes were irreversible. TOTM has mild reversible effects. It is recommended to use safely as an alternative plasticizer.

Keywords: Plasticizers; Immunohistochemisty (Hep Par-1); Liver; Rats

Introduction

Polyvinyl chloride plastics (PVC) are used in the production of a wide array of medical devices and the health care industry including blood bags, plasma collection bags, dialysis bags, catheters, and gloves. This wide range usage of plasticized PVC in devices is attributed to several reasons including flexibility, chemical stability, possibility to sterilize, low cost and wide availability [1]. PVC is a unique polymer because of its ability to accept large quantities of additives to achieve specific qualities. It is a relatively rigid and brittle polymer .Flexibility is achieved through the addition of chemical plasticizers [2,3].

Plasticizers are organic compounds added to PVC to facilitate processing and increase flexibility and toughness in the final product by internal modification of the polymer molecule. There are more than 300 different types of plasticizers described, of which about 50 to 100 are in commercial use. Di (2-ethylhexyl) phthalate (DEHP) is the most commonly used of them [2,4].

DEHP is a lipophilic compound that can be absorbed through skin and lungs by both humans and rodents. The general population is exposed to DEHP mainly through oral rout [5]. The exposure in adults ranges from a few µg up to 25-30 µg /kg bw/day. There are important differences among populations and individuals associated with various dietary habits and lifestyle. Infants and children are exposed to higher levels than adults; on a body weight basis [2,6].There is no covalent bond between DEHP and plastics into which they are mixed. So, they are easily released from products into the environment contaminants [7]. Most animal species metabolize DEHP rapidly into monoethylhexyl phthalate (MEHP) and 2-ethylhexanol (2-EH). By hydrolytic cleavage but at high doses some unmetabolized DEHP can also be absorbed [8].

The biological action of DEHP is very similar to chemicals that are collectively known as peroxisome proliferators (PPs) that associated with increased cellular proliferation and suppression of apoptosis. Reactive oxygen species are thought to be intimately associated with DEHP by the formation of hydrogen peroxide and other oxidants producing DNA damage that may lead to mutations and cancer. Long-term exposure is associated with the development of hepatocellular adenomas and carcinomas [9,10].

As a consequence, the need for alternatives to the existing plasticizers has aroused increasing interest. Trimellitate plasticizers are the alternative for phthalate plasticizers when high temperature applications and high stability are of importance [2].

Trioctyltrimellitate (TOTM) is trimellitate plasticizers which exhibits a low potential of toxicity due to its relative higher molecular weight and bulky side chains. TOTM has unique low leaching and extraction resistance that are required for medical tubing. TOTM leaching properties is about thirty times less than DEHP. TOTM exhibits very limited acute toxicity with an oral LD50 > 2 g/kg, TOTM has low metabolic transformation capacity. Only little percentage is

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Received June 19, 2013; **Accepted** September 26, 2013; **Published** October 11, 2013

Citation: Shehata AS, El-Rehim Mohamed ZA, El-Haleem MRA, Samak MA (2013) Effects of Exposure to Plasticizers Di-(2-Ethylhexyl) Phthalate and Trioctyltrimellitate on the Histological Structure of Adult Male Albino Rats' Liver. J Clin Toxicol 3: 169. doi:10.4172/2161-0495.1000169

metabolized to mono and di-octyl trimellitate (MOTM and DOTM) and 2-ethylhexanol [2]. TOTM have been found to be non-mutagenic. It is not able to bind to peroxisome proliferator-activated receptors (PPARs) [11].

Most of the available previous studies were focused on toxicological and biochemical aspects of exposure to the most widely used plasticizer DEHP. Consequently, an intense research about a biologically inert plasticizer has been initiated. Therefore, the aim of this work is to throw more light on the histological and immunohistochemical changes of adult male albino rat's liver upon exposure to DEHP. Secondly, compare these effects with those related to TOTM exposure and the possibility of improvement after cessation of their administration.

Materials and Methods

Animals

Thirty six adult male albino rats 200-250 g were housed in stainless steel cages at Animal House, Faculty of Medicine, Zagazig University at room temperature, fed standard balanced diet and allowed water ad-libitum. The experiment was performed in accordance with the "Guide for the Care and Use of Laboratory Animals" [12].

Reagents

Di-(2ethylhexyl) phthalate (DEHP): (CAS No. 117-82-7) 99.7% pure, was supplied by Sabic Chemical Company. Corn oil was used as a vehicle.

Trioctyltrimellitate (TOTM): (CAS No. 3319-31-1) 99.5% pure, was supplied by Orientals Chemical Company. Corn oil was used as a vehicle.

Animals grouping

Rats were classified into three equal groups (12 animals each). Group I (Control): Rats were further subdivided into two equal subgroups; subgroup IA: They were given distilled water by oral gavages, 6 days per week for 4 weeks. Subgroup IB: They were given 5ml corn oil by oral gavages, 6 days per week for 4 weeks. Group II (DEHP group): They were treated with DEHP by oral gavages at a dose of 300 mg/ kg 6 days per week for 4 weeks [13].

One day after the last dose half of the animals were sacrificed (DEHP treated subgroup IIA). The other half were left without treatment for another 4 weeks (DEHP recovery subgroup IIB). Group III (TOTM group) were treated with TOTM by oral gavages at a dose of 300 mg/ kg/ day, 6 days per week for 4 weeks [2]. One day after the last dose half of the animals were sacrificed (TOTM treated subgroup IIIA). The other half were left without treatment for another 4 weeks (TOTM recovery subgroup IIIB).

Methods

At the end of the experiment, the rats were anesthetized with 50 mg/ kg bw. Sodium pentobarbital intraperitoneally, intra-cardiac perfusion was done by 2% glutaraldehyde for partial fixation. Liver specimens from right lobe were taken. To prepare paraffin blocks, specimens were immediately placed in 10% buffered formalin. After 10 min, when the tissue was hardened to avoid soft tissue dissipation, About 1 cm^3 specimens were fixed in 10% buffered formalin for 24 h and processed to prepare 5 µm sections stained with haematoxylin and eosin stain to verify the histological details [14]. For immunohistochemical study, the deparaffinised 5 µm paraffin sections on charged slides were used for localization of hepatocyte mitochondrial membrane antigen using hepatocyte paraffin-1 (Hep Par-1) Lamps and Folpe [15] using avidin–biotin-complex (ABC) immunoperoxidase technique. The sections were incubated in hydrogen peroxide for 10 min to block the endogenous peroxidase then incubated with the primary anti-HepPar-1 mouse monoclonal antibody for 60 min at room temperature. The primary antibody used was HepPar-1 mouse monoclonal antibody (clone OCH1E5, CAT. # MS-1810-R7, ready to use, Lab vision). The antibody reaction was detected with HepPar-1 using diaminobenzidine (DAB) as chromogen. Sections were counterstained with haematoxylin for 15 seconds before checked under microscope. Normal liver tissues was used as positive control, while negative control was done using the same tissue (normal liver), omitting the primary antibody [16]. Finally, the Hep Par-1 cytoplasmic sites of reaction were stained brown.

Specimens for electron microscopy were immediately fixed in 2.5% phosphate-buffered glutaraldehyde (pH 7.4). 1 mm^3 specimens from liver were immediately fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for 24 h at 4°C. Then the specimens were washed with the buffer, post fixed in 1% osmium tetroxide in distilled water for 2 h at 4°C. Specimens were dehydrated with ascending grades of ethanol and then put in propylene oxide to prepare Epon-Araldit resin blocks. Ultrathin sections were stained with uranyl acetate and lead citrate [17] and examined and photographed using a JEOL JEM 1010 electron microscope (JEOL Ltd, Tokyo, Japan) in the Electron Microscope Research Laboratory of the Histology and Cell Biology Department, Faculty of Medicine, Zagazig University (Egypt).

Quantitative morphometric measurements were achieved by using the Image analyser (Leica Qwin standard, digital camera CH-9435 DFC 290, coupled to photomicroscope, Germany), Faculty of Medicine, Zagazig University, Egypt. The area percent for HepPar-1 immunoreaction in hepatocytes were measured using magnification 400 with measuring frame area 7286.78 l μm^2 [18]. The data obtained were subjected to statistical analysis using SPSS statistical software (SPSS for Windows, version13.0). Comparison between groups was made using ANOVA, LSD (least significant difference). Data were expressed as mean (±) SD. Results considered significant when P value /0.05.

Results

A-Haematoxylin and Eosin results

The control group liver revealed polygonal classic hepatic lobules. They were in close contact without distinct interlobular connective tissue septa. The portal areas were found at the corners of adjacent hepatic lobules (Figure 1a). Each hepatic lobule was formed of hepatocyte plates radiating from the central vein. Hepatocytes appeared with rounded vesicular nuclei and acidophilic cytoplasm, few cells were binucleated. Blood sinusoids with their kupffer and endothelial cells lining were noticed between the hepatocyte plates (Figure 1b).

DEHP treated subgroup IIA showed variable focal hepatocellular changes representing different degrees of lobular affection in all rats' liver. The dramatically affected ones revealed loss of normal lobular architecture, periportal cellular infiltration, congestion and dilatation of the portal veins. Marked fatty infiltration that replaced most hepatocytes were also detected (Figure 2a). The apparent least affected areas showed vascular congestion with prominent sinusoidal Kupffer cells. Many hepatocytes were binucleated (Figure 2b).

DEHP recovery subgroup IIB showed mild degree of morphological improvement. Focal areas of fatty changes were still observed (Figure

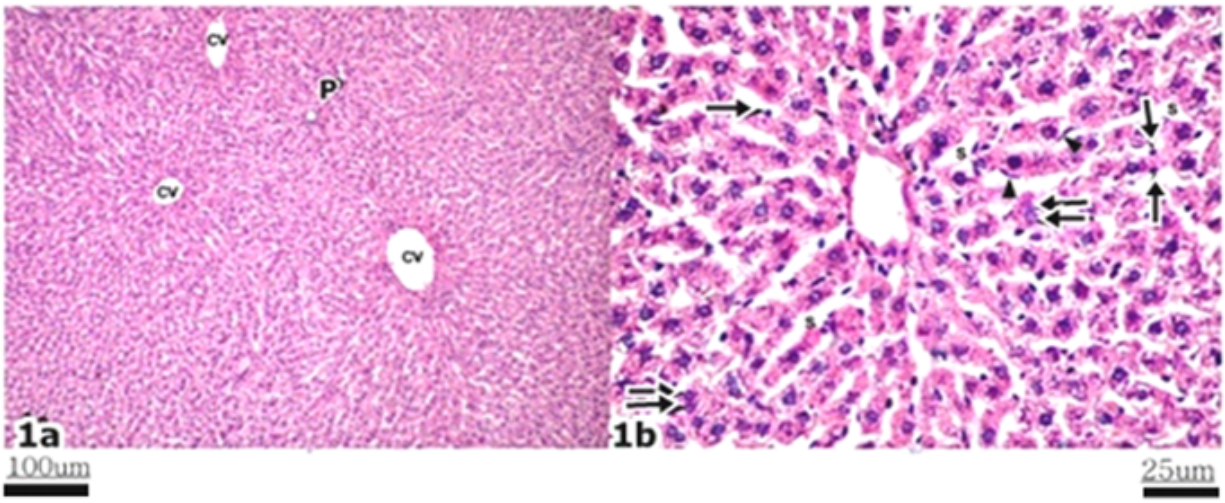


Figure 1: Micrograph of the control group liver. (a): shows classic hepatic lobules with plates of hepatocytes radiating from the central veins (cv). The portal area (P) is noticed at the angles of the adjacent lobules, H&E X100 scale bar = 100µm. (b): shows hepatocytes with rounded vesicular nuclei and acidophilic cytoplasm. Blood sinusoids (S) and their endothelial (arrowhead) and kupffer cells (arrow) are observed in between hepatocytes plates. Few cells are binucleated (double arrows) H&E X400, scale bar = 25µm.

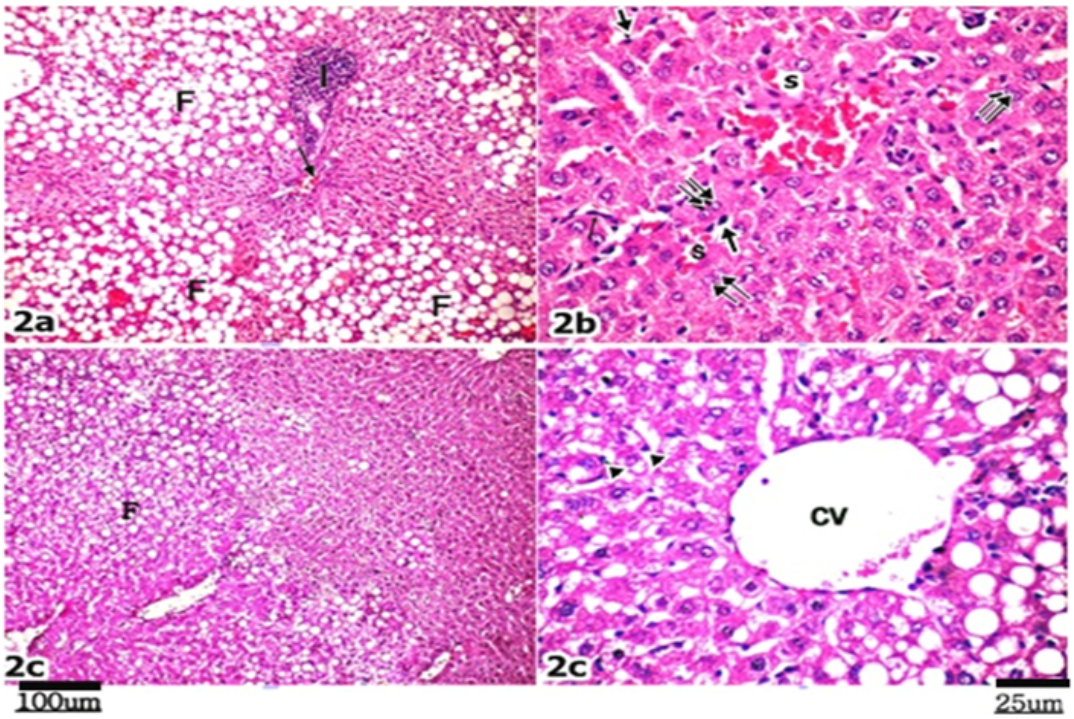


Figure 2: Micrograph of DEHP group (II) liver. (a): Shows marked fatty infiltration (F) within the hepatic parenchyma with loss of lobular architecture. Portal area shows cellular infiltration (I) with congestion and dilatation of the portal vein (arrow), DEHP treated subgroup IIA. (b): Shows congestion of sinusoids (s) with prominent Kupffer cells (arrow). Many hepatocytes were binucleated (double arrows), DEHP treated subgroup IIA. (c): Shows focal areas of fatty changes (F), DEHP recovery subgroup IIB. (d): High magnification of subgroup IIB shows central vein (cv) and dilated blood sinusoids with prominent kupffer cells (arrow). H&E X 400, scale bar = 25µm.

2c). Dilated blood sinusoids with prominent kupffer cells were detected (Figure 2d).

TOTM treated subgroup IIIA showed preserved lobular architecture however generalized hepatic vascular dilatation and congestion was observed. Prominent kupffer cells were noticed in the congested blood sinusoids. Many hepatocytes appeared shrunken (Figure 3a and b).

TOTM recovery group IIIB revealed apparent normal lobular architecture (Figure 3c). Plates of normal hepatocytes showed rounded vesicular nuclei. Slightly dilated blood sinusoids were detected (Figure 3d).

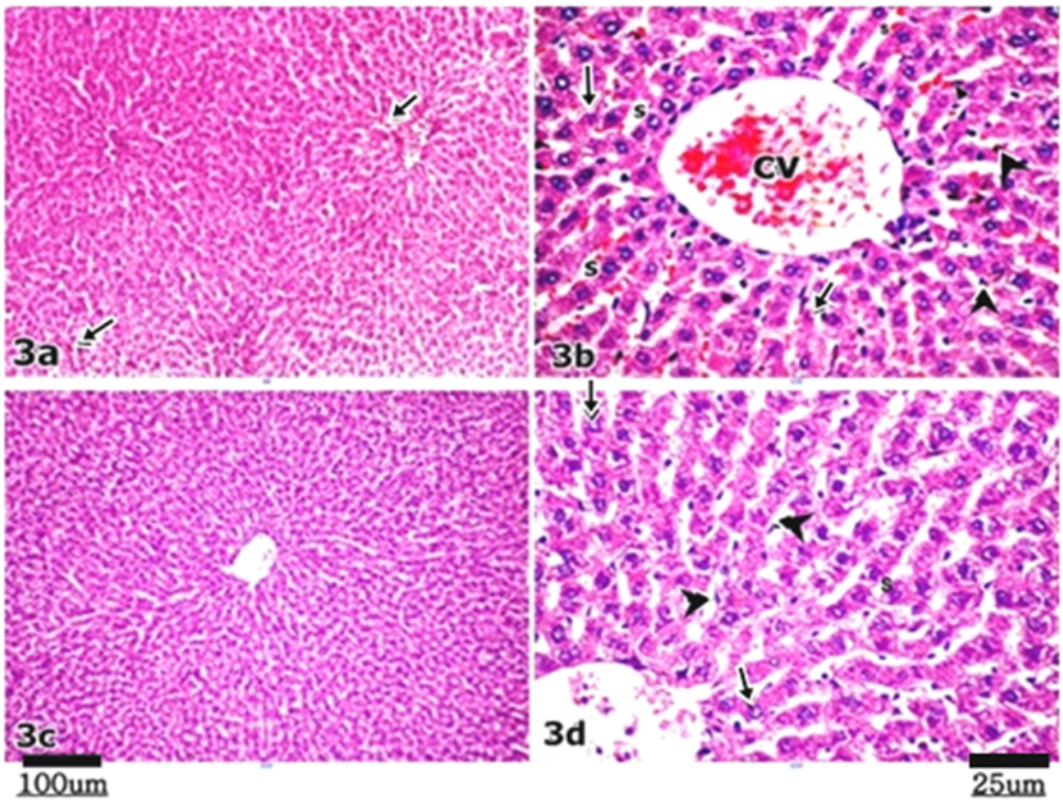


Figure 3: Micrograph of TOTM group (III). **(a):** shows preserved hepatic lobular architecture with dilatation and congestion of the blood vessels (arrow), Subgroup IIIA. **(b):** Higher magnification (a) shows congested central vein (cv) and blood sinusoids (s) with prominent kupffer cells (arrowhead). Hepatocytes appear shrunken (arrow). **(c):** shows hepatic lobule with apparent normal lobular architecture, Subgroup IIIB **(d):** Higher magnification (c) shows plates of normal hepatocytes with rounded vesicular nuclei (arrow) and slightly dilated blood sinusoids (arrowhead).

B- Immunohistochemical results

Immunoperoxidase technique for Hep Par-1 in the control group liver revealed positive Hep Par-1 immunoreaction in the form of scattered granules throughout the hepatocytes cytoplasm (Figure 4).

DEHP treated subgroup IIA revealed heterogeneous granular cytoplasmic immunoreaction of hepatocytes with stronger expression around fatty vacuoles (Figure 5a).

DEHP recovery subgroup IIB revealed moderate positive cytoplasmic immunoreactivity (Figure 5b).

TOTM treated subgroup IIIA revealed positive patchy immunoreactivity. Some hepatocytes showing strong immune reaction, others express weak reaction (Figure 5c).

TOTM recovery group IIIB showed moderate immunoreaction in the cytoplasm of most hepatocytes (Figure 5d).

All the examined groups showed a negative immunoreactive zone around the central veins which were narrow in the control and TOTM subgroups and wide in DEHP subgroups.

C-Electron microscope results

Ultrastructural examination of control group liver showed hepatocytes euchromatic nuclei with prominent nucleoli. The cytoplasm contained mitochondria; some of them appeared in binary fission. Parallel cisternae of rough endoplasmic reticulum (rER) in addition to meshwork of smooth endoplasmic reticulum (sER) tubules and glycogen granules are seen. Intercellular junction's kupffer cells with their indented nuclei were also observed (Figure 6a and b).

DEHP treated subgroup IIA showed variable nuclear profiles. Some hepatocytes contained euchromatic binuclei; others had shrunken nuclei with condensed chromatin. Their cytoplasm contained tubules sER, peroxisomes and pleomorphic lipid globules (Figure 7a). Some hepatocytes showed rarefied cytoplasm and contained clumps of electron dense mitochondria and dilated tubules of sER. The intercellular space contained blood sinusoids, Ito cells with fat droplets and Kupffer cells with indented nuclei and irregular cytoplasmic processes (Figure 7b).

DEHP recovery subgroup IIB showed mild degree of morphological improvement. Some hepatocytes showed euchromatic nuclei, others appeared with irregular nuclear envelopes and clumps of heterochromatin. Their cytoplasm contained dilated sER tubules, cisternae of rER, many lipid droplets, and mitochondria (Figure 7c and d).

TOTM treated subgroup IIIA showed preserved hepatocytes ultrastructure with euchromatic nuclei; some of the had prominent nuclei Their cytoplasm contains few vacuoles, dilated tubules of sER, aggregations of rER cisterae, variable sizes lipid globules, glycogen granules and many mitochondria. Congested blood sinusoids were noticed. Few collagen fibrils were also observed in the space of Disse (Figure 8a and b).

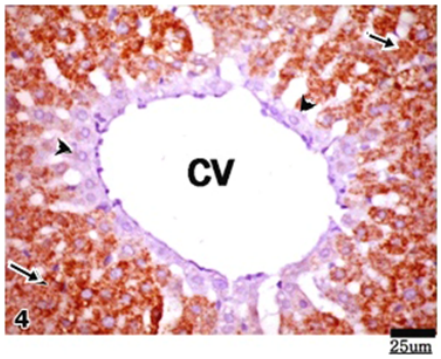


Figure 4: Micrograph of immunoperoxidase technique for Hep Par-1 in the control group liver. Shows positive Hep Par-1 immunoreaction in the form of scattered granules throughout the hepatocytes cytoplasm (arrow). A thin area of negative immunoreaction (arrowhead) around the central vein (cv) is noticed.

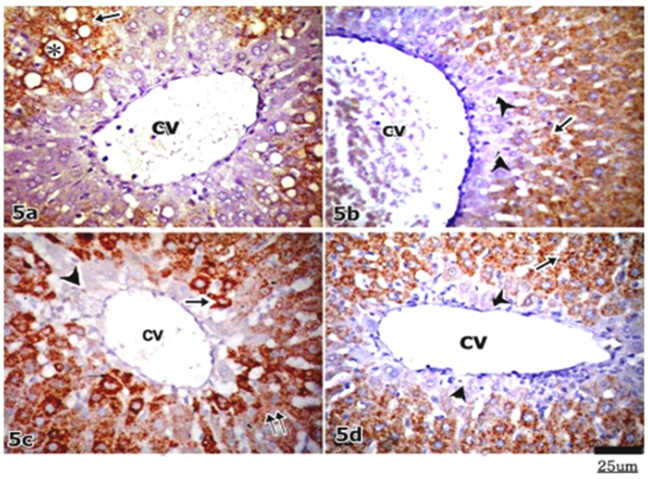


Figure 5: Micrograph of immunoperoxidase technique for Hep Par-1 in both group II & III. (a): Shows heterogeneous granular cytoplasmic immunoreactivity (arrow) of hepatocytes with stronger expression around fatty vacuoles (asterisk). A wide area of negative immune expression (arrowhead) is seen around the central vein (cv), Subgroup IIA. (b): shows hepatocytes with moderate cytoplasmic immunoreactivity (arrow). A relatively wide area of negative immune reaction (arrowhead) is noticed around the central vein (cv). Subgroup IIB. (C): shows patchy immunoreactivity. Some hepatocytes showing strong immune reaction (arrow), others express weak reaction (double arrow). An irregular area of negative immune reaction (arrowhead) around the central vein (cv) is noticed. Subgroup IIIA. (d): shows moderate immunoreactivity in the cytoplasm of most hepatocytes (arrow) except a negative narrow zone (arrowhead) around the central vein (cv), Subgroup IIIB.

TOTM recovery subgroup IIIB revealed apparently normal hepatocytes with euchromatic nuclei; some of them had two prominent nucleoli. The cytoplasm shows many mitochondria, cisternae of rER and tubules of sER (Figure 8c and d).

D- Statistical analysis

The area % of Hep Par 1 immune reaction in subgroup IA is no significantly differ from subgroup IB so subgroup IA is chosen as the control with the other groups. The area % of immune reaction in subgroup IIA was highly significantly decreased with subgroup IA. In subgroup IIB the area % of immune reaction was significant decrease in comparison with the control. Subgroup IIIA was significantly decreased in comparison with the control. On the other hand, area % of immune reaction in subgroup IIIB was significantly increased in comparison to IIB. Moreover, subgroup IIIB was non-significant in comparison with the control (Table 1, Histogram 1).

Discussion

Liver is the main site of plasticizers metabolism and utilization after exposure [19]. DEHP undergo hepatic biotransformation resulting in conversion to more hepatotoxic metabolites and multiple oxidation products readily excreted in bile or urine [8].

The results of this work revealed the livers of the DEHP treated

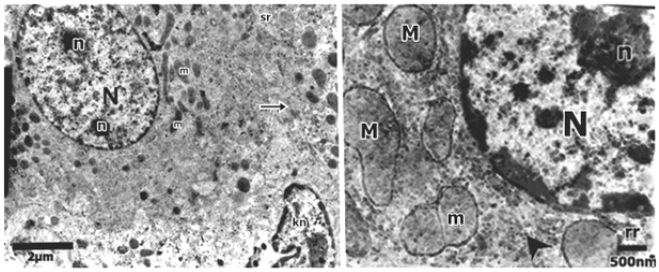


Figure 6: An electron micrograph of hepatocyte in the control group liver. (a): shows hepatocyte with euchromatic nucleus (N) with two prominent nucleoli (n). The cytoplasm shows many mitochondria cisternae of rER (rr) meshwork of smooth endoplasmic reticulum (sr) tubules were distributed in the cytoplasm. Intercellular junction and kupffer cell with indented nucleus were also observed. (b): shows euchromatic nucleus (N) with prominent nucleolus (n). The cytoplasm contains mitochondria (M); one of them appears in binary fission (m). Cisternae of rough endoplasmic reticulum (rr) and glycogen granules (arrowhead) are also seen.

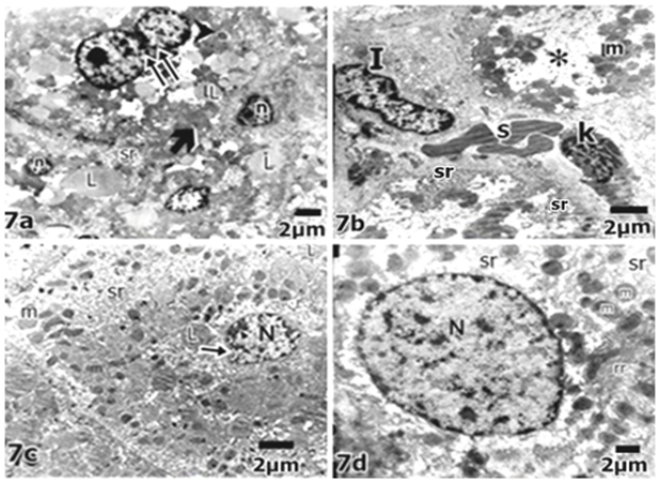


Figure 7: An electron micrograph of hepatocytes in DEHP group (group II). (a): shows variable nuclear profiles; one cell contains euchromatic binuclei (double arrow), while others have shrunken nuclei with condensed chromatin (n). Their cytoplasm contains tubules of sER (sr), peroxisomes (arrowhead), clumps of mitochondria (thick arrow) and lipid globules (L), subgroup IIA. (b): shows rarefied cytoplasm (*) containing electron dense mitochondria (m) and tubules of sER (sr). The intercellular space contains congested blood sinusoid (S), Ito cell (I) with fat droplets and Kupffer cell (k) with indented nuclei, subgroup IIA. (c): shows hepatocyte nucleus (N) with irregular nuclear envelope (arrow) and clumps of heterochromatin. The cytoplasm contained numerous lipid droplets (L), dilated tubules of sER (sr) and mitochondria (m), subgroup IIB. (d): shows hepatocyte with apparently normal euchromatic nucleus (N). The cytoplasm contains dilated sER tubules (sr), cisternae of rER (rr), and mitochondria (m), subgroup IIIB.

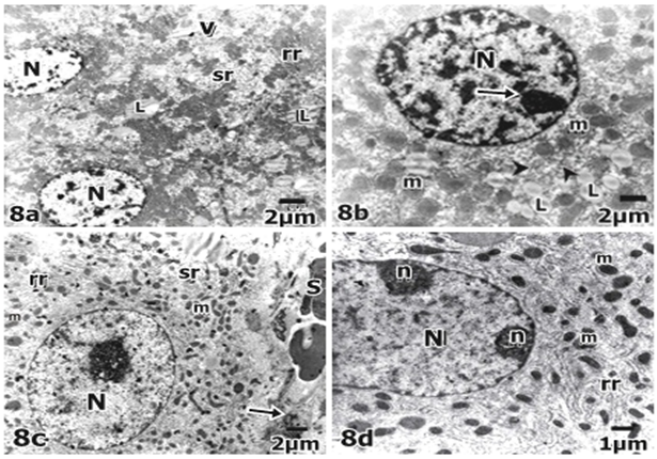


Figure 8: An electron micrograph of hepatocytes in TOTM treated group III. (a): shows euchromatic nuclei (N). The cytoplasm contains few vacuoles (v), dilated tubules of sER (sr), aggregations of rER cisterae (rr) and variable sizes lipid globules (L), subgroup IIIA. (b): Shows hepatocyte with many mitochondria (m), lipid globules (L) and glycogen granules (arrowhead), subgroup IIIA. (c): Shows hepatocyte with euchromatic nucleus (N). The cytoplasm shows many mitochondria (m), cisternae of rER (rr) and tubules of sER (sr). An adjacent congested blood sinusoid (S) is seen. Few collagen fibrils (arrow) are observed in the space of Disse, subgroup IIIB. (d): Shows an apparently normal hepatocyte with euchromatic nucleus (N) and two prominent nucleoli (n). The cytoplasm shows many mitochondria (m) and cisternae of rER (rr), subgroup IIIB.

subgroup / Parameter	IA	IB	IIA	IIB	IIIA	IIIB	F	P
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD		
Area %	81.67 ± 3.98	81 ± 1.67 [n]	61.33 ± 8.14 [b]	70 ± 7.69 [c]	70.5 ± 4.23 [c d]	76.67 ± 3.33 [n]	20.1	0.000**

[n]Non significant when comparing to subgroup IA
[b]Highly significant comparing to subgroup IA
[c]Significant comparing to subgroup IA
[d]Significant comparing to subgroup IIB

Table 1: Comparison of mean area (%) of HepPar-1 immunoreactivity among studied subgroups.

subgroup IIA with variable focal hepatocellular changes representing different degrees of lobular affection. By light microscope examination, increased binucleated profiles were confirmed by electron microscopy. These results were in agreement with previous studies recorded a burst of proliferation in DEHP-treated rats. Elevation in the rate of mitosis of hepatocytes is observed as early as 24 hrs after the initial dose, and it is gradually increased until the fourth week of exposure. Furthermore, a decrease in apoptosis was also suggested to be an important additional molecular event that may affect hepatocytes in DEHP exposed rodents [10]. Kupffer cell-derived oxidants might play a role in signalling increase in cell proliferation caused by peroxisome proliferators (PPs) via a mechanism involving production of tumour necrosis factor TNFα and the transcription factor NF-κB [20].

In the same group some hepatocytes showed shrunken nuclei with condensed chromatin Tasci et al. [21] stated that nucleoplasmic constituents represent the structural counterpart of transcription and processing of messenger and ribosomal RNAs, and therefore constitute fine and highly sensitive indicators of cellular activity.

Hepatocyte cytoplasmic vacuolations were observed in DEHP treated rats. Izunya et al. [22] attributed a similar finding to the oxidative activity of DEHP with subsequent generation of superoxide anion causing lipid peroxidation. Accumulation of these lipid peroxides leads to toxic disintegration of cellular organelles and alteration of membrane permeability with paralysis of Na K pump with subsequent hepatocytes edema.

Histological examination of the same group demonstrated vascular congestion and dilatation. Some investigator postulated that DEHP and MEHP are able to increase nitric oxide production by isolated rat Kupffer cells in a dose-dependent manner with subsequent vascular dilatation [10].

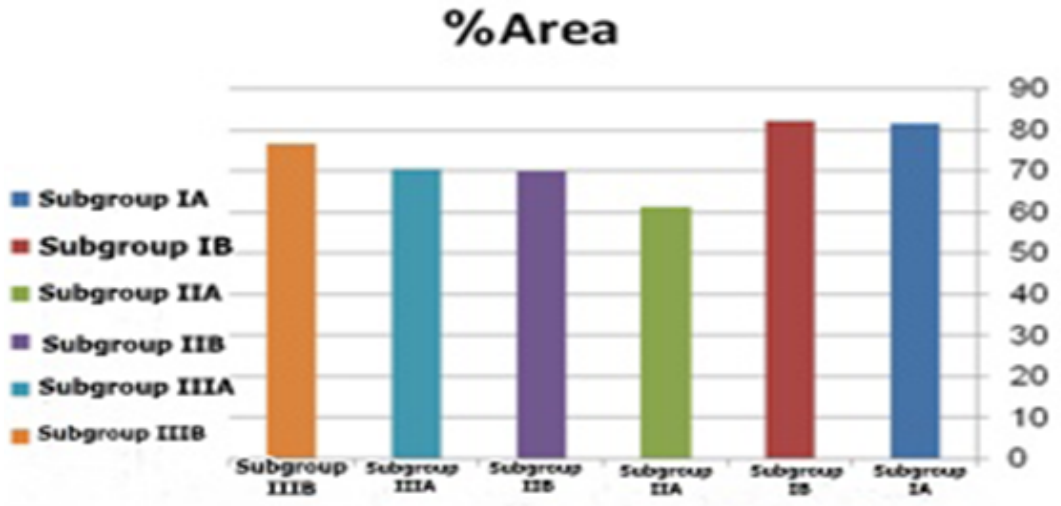
Concerning the hepatocellular fatty infiltration detected in the same subgroup, several investigators reported increase in the activities of the enzymes responsible for fatty acid metabolism (palmitoyl-CoA oxidase, enoyl-CoA hydratase, carnitine acyltransferase and α- glycerophosphate dehydrogenase) by factors as great as 150% in rodents exposed to DEHP. Significant increase in the phosphatidyl-choline and phosphatidyl-ethanolamine with increased total liver lipoid content were reported by Khalik et al. [23]. Moreover, PPARs plays an important role as a modulator of signal molecules that mediate alternations in gene expression of different enzymes involved in lipid homeostasis. Altered hepatic β oxidation pathways lead to medium and long chain fatty acids overload with subsequent increase of acyl coA activity that exceeds the free radicals elimination by catalase resulting in progressive DNA and parenchymal damage [24]. Marked periportal cellular infiltration was observable in DEHP treated subgroup IIA. These hepatitis indicator changes may be attributed to that phthalates promote cytokine IL-6 and IL-8 production in the human epithelial cell line [25]. Both cytokines are key modulators of inflammatory response that are produced by activated monocytes and neutrophils [26].

In the current study, peroxisomes were detected in the cytoplasm of hepatocytes of DEHP treated subgroup IIA. This observation was in agreement with the finding of Rusyn et al. [10]. They stated DEHP induced activation of PPARs and agonistic interaction of DEHP and its metabolite MEHP with these receptors lead to oxidative stress and generation of electrophonic free radicals. However, limited evidence indicated that liver cells from humans and from some other non-rodent species were relatively non-responsive to induction of peroxisome proliferation by phthalates, the possibility that such agents might pose a carcinogenic risk to humans could not be ruled out [27].

Electron microscope examination of DEHP subgroup IIA revealed clumps of electron dense mitochondria. Similar results were observed by many authors who attributed these changes to the presence of crystalline inclusions which are either over-products of mitochondria or crystallization of mitochondrial enzymes resulting from disordered hepatic metabolism [28,29].

In the present work, examination of liver sections of DEHP recovery subgroup IIB showed variable degrees of improvement according to severity of changes observed in DEHP treated group. These results may be attributed to down-regulation of PPARs or peroxisomal enzymes besides the reduction of hyperplastic hepatocytes through apoptosis [30].

In the present study, light microscope examination of the liver sections of TOTM treated subgroup IIIA showed preserved lobular architecture however generalized hepatic vascular dilatation and congestion was observed. Prominent kupffer cells were noticed in the congested blood sinusoids. Many hepatocytes appeared shrunken. These were in accordance with the changes reported by [2]. These changes could be attributed to kupffer cell activation with subsequent



Histogram 1: mean area (%) of HepPar-1 immunoreactivity.

increase of nitric oxide production and subsequent vascular dilatation [20]. Previous studies revealed that TOTM no observed adverse effect level (NOAE)L was 1000 mg/kg/day [31].

Transmission electron microscope of the TOTM treated rat's showed preserved hepatocytes ultrastructure with euchromatic nuclei. Their cytoplasm contains few vacuoles, variable sizes lipid globules and many mitochondria. Tasci et al. [21] stated that disorganization of nuclear content as margination and clumping of chromatin in saline treated animals may be morphological evidence of injury in the nucleus. Kambia et al. [11] reported that, following TOTM administration, high rate of new mitochondrial membrane formation is due to increased endogenous mitochondrial protein synthesis in addition to increased rate of membrane phospholipid synthesis by about 20%.Sections of the same group showed dilated tubules of sER of hepatocytes. Since sER plays a prominent role in the metabolic transformation of drugs and toxins. It was stated that the oxidative stress imposes an increased functional strain on hepatocytes resulting in dilatation of this organelle and increased its enzymatic production. Moreover, hepatocellular hypoxia plays another role leading to sER tubular dilatation [21].

Compared with DEHP treated group, these results suggested the far less hepatocellular damaging effect of TOTM. This was explained by some investigators who compared the potential molecular interaction between DEHP and TOTM with PPARα and PPARγ binding sites. They discovered that DEHP binds to both PPAR subtypes while TOTM is unable to fit in the binding site of either receptor due to its large molecular weight and heavy side chains [11]. So, TOTM minor hepatic histological changes can be explained by PPAR independent mechanisms and sequels of kupffer cells activation which proven to be PPAR-independent

In the present study, light microscope examination of liver sections of TOTM recovery subgroup IIIB revealed relative restoring of general hepatic lobular architecture with plates of normal hepatocytes. Slightly dilated blood sinusoids were detected. Ultrastructurally, hepatocytes showed apparently normal euchromatic nuclei; some of them had two prominent nucleoli. The cytoplasm shows many mitochondria, cisternae of rER and tubules of sER. These findings are in accordance with that reported by Zimmermann [32]. They emphasized that regeneration of hepatocyte populations is chiefly regulated by hepatocyte growth factor and transforming growth factor-α. This reconstruction process goes in line with a remodelling response, resulting in regeneration of normal hepatic lobule**.** The proliferation of hepatocytes advances from periportal to pericentral areas of the lobule, as a wave of mitosis. Replacement of lost hepatic mass is mediated through proliferation of mature adult hepatocytes and the other hepatic cell types. Previous studies suggest that lobular reorganization taking place for several weeks, and eventually liver histology becomes indistinguishable from the original [33].

In the present work, immunohistochemical staining for Hep Par-1 of control group revealed positive immunoreaction appeared as scattered granules throughout the hepatocytes cytoplasm without zonal preference. This was in accordance with previous investigators who approved the antigen for Hep Par-1 antibody is Carbamoyl Phosphate Synthetase 1 (CPS1) which is an abundant hepatocellular protein predominantly localizes in the mitochondria. CPS1 is a rate-limiting enzyme in urea cycle. Hep Par-1 has a high sensitivity and specificity of Hep Par 1 for hepatocytes. There is no immunohistochemical staining of bile ducts or other hepatic non-parenchymal cells [34,35].

All the examined groups showed a negative immunoreactive zone around the central veins which were narrow in the control and TOTM group (IIIA&B) and wide in DEHP group (IIA&B). Previous studies stated that the relative cellular hypoxia that affects mitochondrial metabolic pathways in the layer of hepatocytes adjacent to the central vein might explain the negative expression of this area [36].

DEHP treated subgroup IIA revealed heterogeneous granular cytoplasmic immunoreaction of hepatocytes with stronger expression around fatty vacuoles. The area % of immune reaction in this group was highly significantly decrease compared to control group. Previous studies reported that Hep Par 1 expression is the histological evidence of hepatocellular origin of hepatocellular carcinomas [37]. In this study, altered mitochondrial metabolic pathways by both PPARs dependent and independent mechanisms besides altered gene expression of mitochondrial enzymes may be responsible for this heterogeneous immune-expression**.**

Positive immunoreaction was noticed in hepatocytes cytoplasm around fat globules in group (IIA&B). It was reported that Hep Par 1 immunoreactivity in fatty livers was maintained in the cytoplasm

adjacent to fat vacuoles. This finding is of great importance as it is an indicator that the hepatic fatty infiltration in both groups is endogenous hepatocellular in origin and not due to Ito cell proliferation and infiltration [15].

TOTM treated subgroup IIIA revealed positive patchy immunoreactivity which was significantly decreased in comparison with group IA. TOTM recovery subgroup IIIB showed moderate immunoreaction in the cytoplasm of most hepatocytes. The area % of immune reaction in subgroup IIIB was significantly increased in comparison to IIB while it was non-significant with group IA. These results support the light microscopic and ultrastructural findings which suggested that TOTM mild hepatic histological changes were reversible four weeks after cessation of treatment. These results can be explained by the presence of many mitochondria in hepatocytes.

In conclusion, the present work revealed that the plasticizer DEHP induced serious histological, ultrastructural and immunohistochemical abnormalities on adult male albino rats' liver, some of these hepatic changes were irreversible. On the other hand, the same study on TOTM has proven the mild effects of this new plasticizer. So it could be used as an alternative plasticizer with less adverse impacts. Further studies are recommended to clarify the safety of TOTM on long-term exposure.

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