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Histological and Ultrastructure Changes Induced by Di {2-ethylhexyl} Phthalate (dehp) in the Alveolar Tissue of Adult Albino Rats and the Possibility of Recovery

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Abstract

Background: DEHP is a commonly used phthalate plasticizer in polyvinylchloride (PVC) formulations used by many medical devices. It is known to be released with time into the biological fluids or redistributed into various tissues increasing the risk of certain health hazards that include developmental anomalies, reproductive and respiratory health effects.

Objective: To investigate the histopathological effects of DEHP on the lung alveolar tissues and the possibility of recovery after stoppage of DEHP administration in adult albino rats.

Methods: Thirty adult male albino rats were divided equally into three groups, received the following via orogastric intubation once daily for 2 weeks: Group I (control group): received normal saline. Group II (DEHP treated group): received DEHP dissolved in normal saline. Group III (DEHP recovery group): received DEHP as group II and then left untreated for another 2 weeks. The right lung from each animal was dissected, cut into small pieces. Some specimens were processed for paraffin sections to be stained with Haematoxylin & Eosin and Masson's trichrome, others were processed for semi-thin sections, and ultrathin sections to be examined with Transmission Electron Microscope.

Results: The alveolar tissue in DEHP treated group showed marked increase in the thickness of the interalveolar septa with collagen deposition and inflammatory cellular infiltration associated with many collapsed alveoli. Most of type II pneumocytes showing necrotic changes in the form of vacuolated or deeply acidophilic cytoplasm with karyorrhetic or pyknotic nuclei. Additionally, there was interstitial hemorrhage. We found a statistically significant increase in the thickness of the interalveolar septa, number of type II pneumocyte, number of alveolar macrophages/field and the area percentage of collagen fibers when compared to control group. Ultrastructurally, type II pneumocytes showed degenerative changes in the form of cytoplasmic vacuolation and destruction of lamellar bodies and mitochondria. However, alveolar tissue changes showed mild improvement after drug stoppage.

Conclusion: DEHP has toxic effect on the alveolar tissues which was not totally improved after its withdrawal.

Keywords: Plasticizer; DEHP; Alveolar tissue; Electron microscope

Introduction

Phthalates are materials with a wide spectrum of industrial applications. High molecular weight phthalates are primarily used as plasticizers in the manufacture of flexible vinyl. Low molecular weight phthalates are used in personal-care products, as solvents for cellulose acetate, and in making lacquers, varnishes, and coatings [1]. A quarter of the plasticizers produced are made of di-(2-ethylhexyl) phthalate (DEHP). DEHP is a commonly used phthalate plasticizer in polyvinylchloride (PVC) formulations. It has a wide variety of applications from cosmetics and food packaging to medical devices, and it makes PVC tubing soft and flexible. PVC is used in a range of medical devices from intravenous (IV) fluid containers, blood bags to medical tubing (enteral feeding tubes, respiratory tubing) and catheters [2].

Because Di {2-ethylhexyl} phthalate (DEHP) is not chemically bound to the polymer in a PVC medical device and it is highly lipophilic, it can be released when the device is heated or it can leach out when the device comes into contact with certain media, such as blood or other lipid-containing solutions[2,3]. The daily human exposure to DEHP worldwide is estimated to be in range from 3-30 μg/kg/day in adults, and up to 85 μg /day in children , while exposure in the medical settings (via plastic tubing, IV bags, etc.), may increase up to 2 mg/day. DEHP exposures in the medical setting are of particular concern because the amount of exposure can be substantial and because those exposed, such as premature infants and other neonates or adults with life threatening illnesses, may be particularly vulnerable to the effect of toxic chemicals [2,4].

DEHP can be absorbed from the gastrointestinal tract, the lungs and through the skin [5]. It is rapidly metabolized by pancreatic lipase to mono-2-ethylhexyl phthalate (MEHP) and 2-ethylhexanol. The MEHP is immediately further oxidized into a variety of polar products in vivo as well as in vitro [6]. The well characterized reproductive toxicity and hepatocarcinogenecity exerted by DEHP in rodents have been suggested to be caused by MEHP [7-9].

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Pregnant female rats dosed with DEHP in their diet during their last week of pregnancy and for two days following birth had pups with a substantial decrease in the number of lung parenchymal airspaces, a significant increase in the airspace mean size, and an increase in the number of type II pneumocytes [10]. Similar "alveolar simplification" in addition to increased epithelial and mesenchymal cell proliferation was also reported in the distal lung parenchyma of rat pups treated under similar conditions and they referred these changes to unknown mechanisms [11].

As mentioned earlier, most of the available data are related to fetal lung changes due to DEHP exposure, because of that; the current study was designed to investigate the histological changes in adult respiratory alveoli induced by DEHP and the possibility of recovery after its withdrawal using light and electron microscopy.

Materials and Methods

Animals

30 adult male albino rats weighing 200-220 gm were used in this study. They were maintained at a 12-h day and 12-h night cycle. They were housed in clean properly ventilated cages under the same environmental conditions with free access to food and water for an acclimating pre-experimentation period of one week and throughout the whole period of the experiment.

Study groups

The animals were divided into three groups (10 rats each). All animals received the following via orogastric intubation once daily for 2 weeks:

Group I (The control group): 2.85 ml/kg BW/day of normal saline.

Group II (The DEHP treated group): 2.85 mg/kg BW/day of DEHP [12] dissolved in normal saline (purchased from Aldrich chemical company, Germany).

Group III (The DEHP recovery group): Animals treated with 2.85 mg/kg BW/day of DEHP dissolved in normal saline for 2 weeks, and then left free without DEHP treatment for another 2 weeks.

At the end of the experiment, all rats were sacrificed by decapitation. Specimens from right lung were processed for light and transmission electron microscopes examination.

Histological study

For light microscope, the specimens were fixed in 10% formalin for 12 hours to prepare paraffin blocks. Sections (5 μm) were prepared and stained by H&E stain and Masson's trichrome [13]. For transmission electron microscope, the specimens were immediately fixed in 2.5% glutaraldhyde solution for 24 hours at 4°C, processed for preparing semithin sections (0.5 μm) to be stained by 1% toluidine blue and examined with light microscope. Then ultrathin sections (70-90 nm) were prepared and stained with lead citrate followed by uranyl acetate [14]. Stained sections were examined with a JEOL 1010 Transmission Electron Microscope in the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University [14].

The following histological changes were assessed in H&E stained sections:

1. Degeneration: (vacuolated or lightly stained cytoplasm), deeply esinophilic cytoplasm, necrosis and proliferation (mitotic figures), in type II pneumocyte.
2. Alveolar septal changes such as thickening, decrease or increase of connective tissuefibers and pulmonary interstitial changes (mononuclear cellular infiltration).
3. Vascular changes such as, congestion of pulmonary vessels and hemorrhage.

Morphometric analysis

The image analyzer (Super eye-Heidi soft) in Histology department, Faculty of Medicine, Suez Canal University was used to obtain the following morphometric data:

- The mean thickness of interalveolar septa using H&E stained sections at x400 magnification.
- The mean number of type II pneumocytes/ field using oil immersion lens in H&E stained sections.
- The mean number of alveolar macrophages/ field using oil immersion lens in toluidine blue stained semithin sections.
- The mean area percentage of collagen fibers using Masson's trichrome stained sections at x400 magnification.

The previous measurements were estimated in five non-overlapping fields/section in five serial sections/rat from each animal in each group.

Statistical analysis

All statistical analysis was performed using the statistical software package SPSS 15.0 for Windows® (SPSS Inc., Chicago, IL, USA). The obtained data were expressed as mean ± standard deviation and analyzed using analysis of variance (ANOVA). Statistical significance level was defined as $P<0.05$.

Results

The control group (group I)

H&E stained sections of the alveolar tissue showed normal histological architecture; numerous clear alveoli with thin interalveolar septa, and clearly seen alveolar sacs. The alveolar epithelium showed extremely flattened type I pneumocytes with densely stained flattened nuclei and very thin cytoplasm; and cuboidal type II pneumocytes with large dark stained rounded nuclei, which were commonly located near the angles between neighboring alveolar septa (Figure 1).

Masson's trichrome stained sections revealed normal distribution of thin collagen bundles in pulmonary interstitium around alveolar sacs and in interalveolar septa (Figure 2).

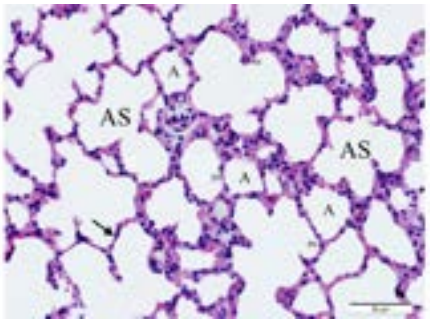

Figure 1: A photomicrograph of a control rat alveolar tissue showing normal alveolar sacs (AS), alveoli (A) and partially thin interalveolar septum (arrow) lined by type I pneumocyte (PI) and type II pneumocyte (PII). (H & E x400).

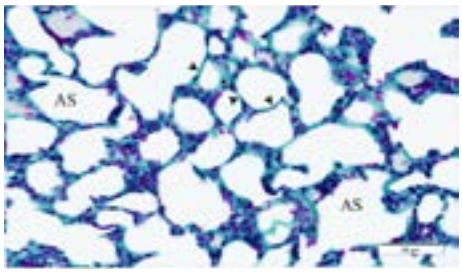

Figure 2: A photomicrograph of a control rat alveolar tissue showing normal distribution of collagen fibers in pulmonary interstitium around alveolar sacs (AS) and in a partially thin interalveolar septa (arrow head). (Masson's trichrome x 400).

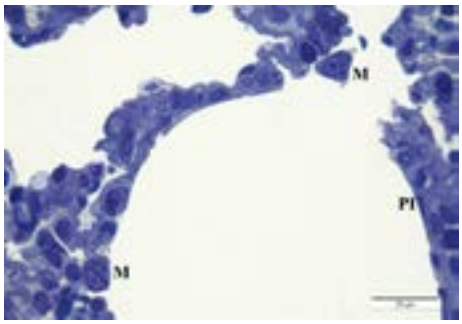

Figure 3: A photomicrograph of a semithin section of a control rat alveolar tissue showing pulmonary alveoli which are lined by type I pneumocyte (PI) and alveolar macrophages also shown (M). (Toluidine blue x1000).

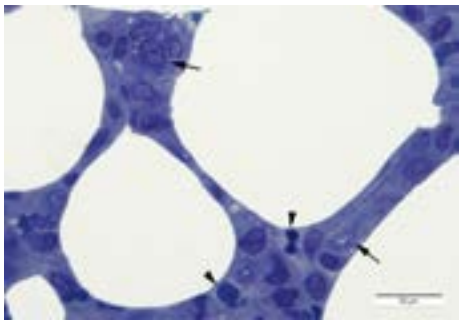

Figure 4: A photomicrograph of a semithin section of a control rat alveolar tissue showing type II pneumocyte (arrow), some RBCs inside pulmonary vessels (arrow head). (Toluidine blue x1000).

Touidine blue stained semithin sections showed thin interalveolar septa that lined by alveolar epithelium which were: flattened squamous cells, type I pneumocytes with its deeply stained nuclei and type II pneumocytes with its irregular cuboidal shape, large dark stained rounded nuclei, with prominent nucleolus and vacuolated cytoplasm. Alveolar macrophages were seen with its characteristic eccentric kidney-shaped nuclei, irregular outlines and cytoplasmic granules (Figure 3 and 4).

Transmission electron microscopic results

Ultra thin sections of alveolar tissue of the control group showed empty and clear alveolar spaces. The walls of the alveoli were lined with type I "squamous" epithelial cells and type II epithelial cells. Large elongated type I pneumocytes showed their morphologic characteristics flattened nucleus and large flat surfaces lacking microvilli. On the other hand, type II pneumocytes appeared much larger in size, with large rounded nuclei showing peripheral condensed chromatin. Their cytoplasm contained numerous dense mitochondria; membrane bounded lamellar bodies containing electron- dense secretions, surfactant. Short microvilli were seen projecting from type II penumocytes borders (Figure 5).

Also, alveolar macrophages were evident by their characteristic indentable nuclei, short microvilli and lysosomes with different densities (Figure 6).

Figure 5: Transmission electron micrograph of a control rat alveolar tissue showing a type II pneumocytes with a large nucleus. Its cytoplasm contained numerous dense mitochondria (m), lamellar bodies (arrow head). Short microvilli were also shown (arrow). (TEM x 15000).

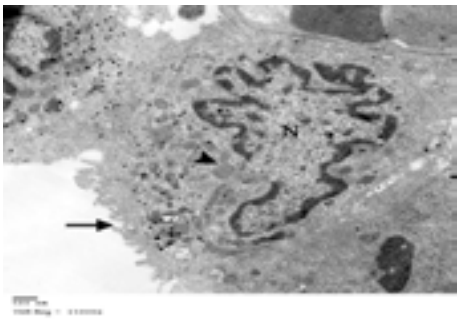

Figure 6: Transmission electron micrograph of a control rat alveolar tissue showing alveolar macrophage with short microvilli (arrow), some primary lysosomes (arrow head) and indentable vesicular nucleus. (TEM x 15000).

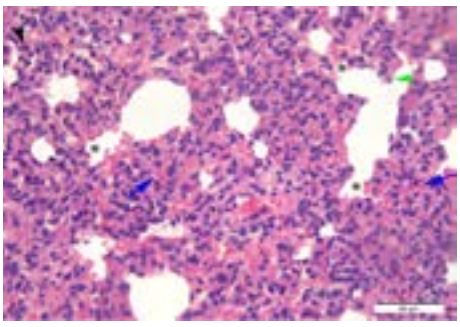

Figure 7: A photomicrograph of a DEHP treated rat alveolar tissue showing marked thickening of interalveolar septa, interstitial hemorrhage (blue arrow), extravasated RBCs (*), necrotic type II pneumocytes with karyorrhectic nuclei (green arrow head). Some type II pneumocytes with mitotic figures are also shown (black arrow head). (H & E x400).

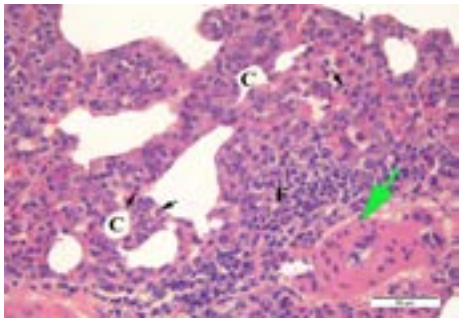

Figure 8: A photomicrograph of a DEHP treated rat alveolar tissue showing collapsed alveoli (c). Thickened pulmonary vascular wall (green arrow), inflammatory cell infiltration (F) as well as necrotic type II pneumocytes with pyknotic nuclei (black arrow) are also shown. (H & E x400).

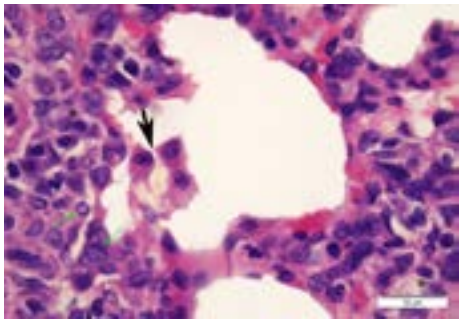

Figure 9: A photomicrograph of a DEHP treated rat alveolar tissue showing desquamated type II pneumocytes (black arrow), cells with chromatin margination (green arrow). Some mitotic figures are shown (*). (H & E x1000).

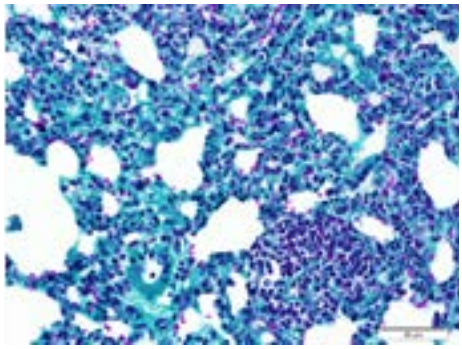

Figure 10: A photomicrograph of a DEHP treated rat alveolar tissue showing marked deposition of collagen fibers in thick interalveolar septa and inflammatory cell infiltration. (Masson's trichrome x 400).

The DEHP treated group (group II)

H&E stained sections of the alveolar tissue in DEHP treated group showed marked increase in the thickness of the interalveolar septa with interstitial hemorrhage (Figure 7). Inflammatory cellular infiltration; mainly perivascular, collapse of some alveoli was also noticed (Figure 8). At the same time, there were apparent changes in type II pneumocyte; which showed necrotic changes in the form of vacuolated or deeply eosincophilic cytoplasm with pyknotic or karyorrhectic nuclei, enlarged nuclei with chromatin margination (Figure 7 and 8). Some nuclei showed mitotic figures (Figure 7 and 9). Some desquamated type II pneumocytes were found in the alveolar spaces. Interstitial hemorrhage with extravasated red blood corpuscles in the alveolar spaces and in the pulmonary interstitium were also seen (Figure 7). Comparing this group to control group, there was highly statistically significant increase in the number of type II pneumocyte and the thickness of the interalveolar septa ($P < 0.001$) (Table 1).

Masson's trichrome stained sections revealed marked increase in deposition of collagen bundles in pulmonary interstitium around alveolar sacs and in interalveolar septum (Figure 10). This was confirmed by a highly significant increase in the mean color area percentage of collagen fibers in this group compared to group I (the control group) ($P<0.001$) (Figure 11).

Study groups	Mean thickness of interalveolar septa ± SD	Mean number of Type II pneumocytes/	Mean number of alveolar macrophages/
Group I: (control group)	8.89 ± 0.363	3.8 ± 0.979	3.8 ± 1.032
Group II: (DEHP treated group)	31.24 **± 3.322	9.1**± 1.3	9.5**± 1.581
Group III: (DEHP recovery group)	17.27 *● ± 0.853	5.5 *● ± 0.806	6.1* ● ± 1.370

*indicates significant difference from the control group (P < 0.05)
**indicates highly significant difference from the control group (P < 0.001)
● indicates significant difference from the DEHP treated group (P < 0.05)
Table 1: Thickness of interalveolar septa, number of type II pneumocytes and alveolar macrophages/field in the different studied groups expressed as mean±SD.

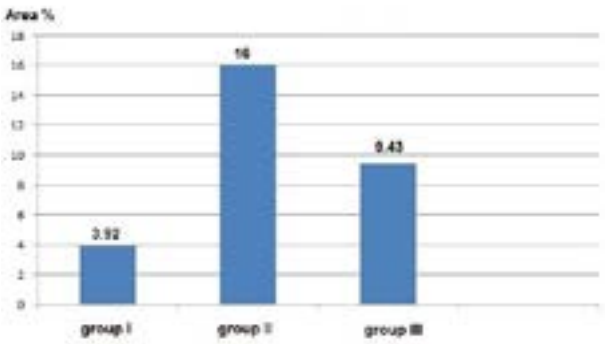

Figure 11: Mean color area percentage of collagen fiber in the different studied groups.

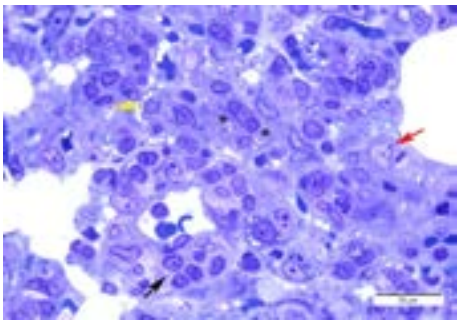

Figure 12: A photomicrograph of a semithin section of a DEHP treated rat alveolar tissue showing loss of normal alveolar architecture in thickened interalveolar septum, increased number of alveolar macrophages surrounding collapsed alveoli (black arrow). Large numbers of type II pneumocyte are shown, some with chromatin margination (yellow arrow); karyolytic nuclei (*) and enlarged pale nucleus (red arrow). (Toluidine blue x1000).

Touidine blue stained sections showed thickened interalveolar septa, disrubted alveolar architecture, increased number of type II pneumocyte showing chromatin margination and/or karyolytic nuclei. Increased number of macrophages surrounding collapsed alveoli was evident. There was highly statistically significant increase in the number of alveolar macrophages/field when compared to group I (P<0.001); (Figure 12) (Table 1).

Transmission electron microscopic results

Using TEM, type II pneumocytes showed cytoplasmic vacuolation and destruction of mitochondrial cristae (Figure 13). Lamellar bodies appeared empty and the short microvilli were lost from the free surfaces of most of type II pneumocytes (Figure 12 and 14). Some nuclei were condensed with loss of normal chromatin pattern (Figure 14); others appeared dividing (Figure 12). No changes were observed in type I pneumocytes which appeared unaffected. The alveolar macrophages were numerous and showed absence of lysosomes with loss of microvilli (Figure 15).

The DEHP recovery group (group III)

H&E stained sections of the alveolar tissue in DEHP recovery group showed less changes compared to group II. The interalveolar septa were not as thick as in the group II. There was less inflammatory cellular infiltration (Figure 16). Some alveoli showed extravasated blood cells in their lumen. Type II pneumocytes showed cytoplasmic degenerative changes and some necrotic type II pneumocyte were also found (Figure 17). There was statistically significant increase in the number of type II pneumocyte and the thickness of the interalveolar septa when compared to group I (P < 0.05) and there was statistically significant decrease when compared to group II (P < 0.05); (Table 1).

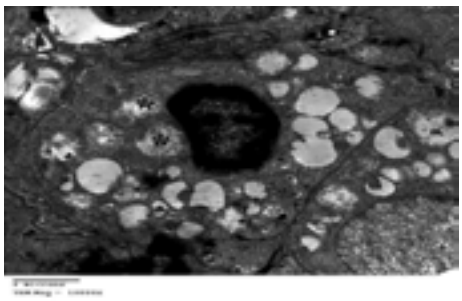

Figure 13: Transmission electron micrograph of a DEHP treated rat alveolar tissue showing type II pneumocyte with pyknotic nucleus, loss of the short microvilli, disrupted mitochondria (*), emptiness of lamellar bodies. (TEM x10000).

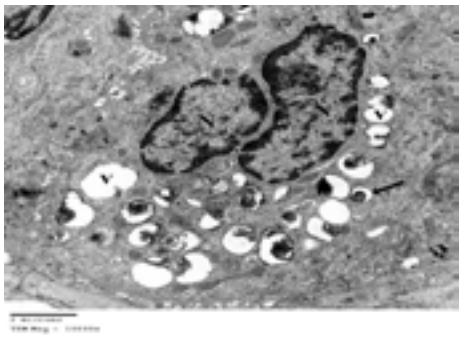

Figure **14:** Transmission electron micrograph of a DEHP treated rat alveolar tissue showing dividing pneumocyte type II with distorted lamellar bodies ( ), or vacuoles (v). (TEM x 10000).

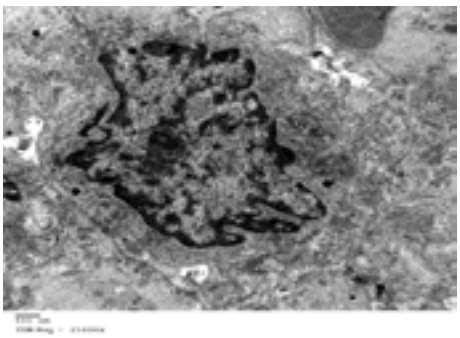

Figure 15: Transmission electron micrograph of a DEHP treated rat alveolar tissue showing a macrophage with complete loss of lysosomes and the microvilli. (TEM x 15000).

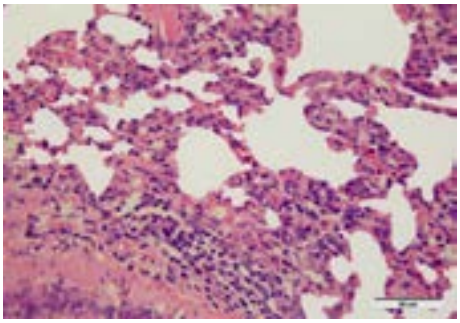

Figure 16: A photomicrograph of a DEHP recovery rat alveolar tissue showing dilatation of most alveoli, few collapsed alveoli, with less inflammatory cell infiltration. (H & E x400).

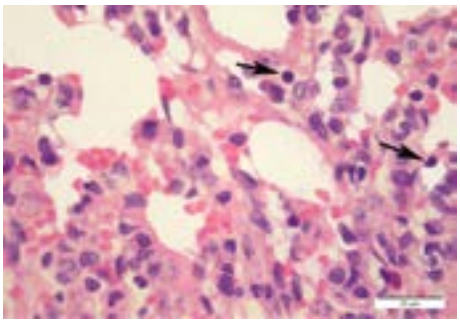

Figure 17: A photomicrograph of a DEHP recovery rat alveolar tissue showing extravasated blood cells in alveolar lumens, with less inflammatory cell infiltration. Some necrotic type II pneumocytes with pyknotic nuclei are shown (black arrow). (H & E x1000).

Masson's trichrome stained sections revealed increased deposition of collagen bundles in the interalveolar septum and also in the wall of dilated congested pulmonary vessel wall when compared to control group (Figure 18). There was statistical significant increase in the mean color area percentage of collagen fibers in this group compared to group I (P < 0.05) at the same time, there was statistical significant decrease when compared to group II (P<0.05); (Figure 19).

Touidine blue stained sections showed less thickened interalveolar septa with some type II pneumocyte showing chromatin margination.

Alveolar macrophages were more than that seen in control group. There was statistically significant increase in the number of alveolar macrophages/field when compared to group I ($P < 0.05$) and there was statistically significant decrease in their number when compared to group II ($P < 0.05$); (Figure 20) (Table 1).

Transmission electron microscopic results

Ultra thin sections of alveolar tissue of the DEHP recovered group showing mild improvement compared to DEHP treated group. Some of type Il pneumocytes regained their normal appearance as their nuclei showed normal pattern of chromatin distribution, and partially empty lamellar bodies (Figure 21). Some alveolar macrophages were seen in the interalveolar septa still with loss of their lysosomes and short microvilli (Figure 11).

Discussion

DEHP is known to be released with time into the biological fluids and redistributed into various tissues causing a harmful effect on the health [10,15].In laboratory animals, DEHP administration showed toxic effect on several organs, particularly the lung [11]. The present study examined the histological changes in rat`s lung treated with di(2-ethyl-hexyl) phthalate (DEHP) .

In agreement with the data obtained from several studies [16,17], our results showed that the lung of rats was found to be sensible to DEHP toxicity. The alveolar tissues from DEHP-treated rats revealed significant increase in the mean thickness of alveolar septa (31.24 **± 3.322), the number of macrophages (9.5**± 1.581), type II pneumocyte (9.1**± 1.3) and the mean area percentage of collagen fibers deposition; when compared with the normal animal tissues. Also, the alveolar tissue from DEHP-treated rats showed variation of the alveolar shape and size, where some were obliterated and others were dilated; but all revealed thickened wall, infiltrated with more inflammatory cells. Also, inflammatory cellular infiltration had been noticed around the thickened pulmonary blood vessels. These changes are commonly described with severe airway injury, fibrosis and chronic inflammatory lung diseases [18-20]. This may be the result of phthalate-induced increases in oxidative stress or inflammation in animal tissues. Oxidative stress and inflammation are known to be interrelated, and oxidative stress-stimulated inflammation has been proposed to be part of the etiologic pathway for DEHP-induced tumorigenesis [21]. However, DEHP is supposed to act through peroxisome proliferator-activated receptors (PPARs) that were over-expressed in cultured type II pneumocytes. Overexpression of peroxisome proliferator-activated receptor-γ (PPARγ) in type II pneumocytes induces inflammatory reaction, inflammatory cell infiltration and T-cell suppression in the lung tissue [17].

Our results also indicated increase in the number of type II pneumocytes in treated animals with disturbed lamellar bodies which was also found in a similar study [10]. Surprisingly, a similar increase in type II pneumocytes has occasionally seen in children affected with hyaline membrane disease [18], a pathology to which DEHP leakage from PVC tubes has been suggested to contribute [22].The increased number of type II pneumocytes might depend on delayed differentiation

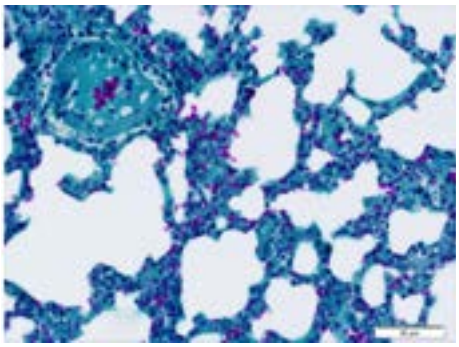

Figure 18: A photomicrograph of a DEHP recovery rat alveolar tissue showing moderate deposition of collagen fibers in the wall of pulmonary blood vessel and in partially thick interalveolar septum. (Masson's trichrome x 400).

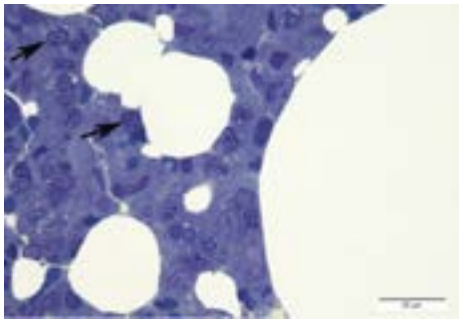

Figure 19: A photomicrograph of a semithin section of a DEHP recovery rat alveolar tissue showing partially thickened interalveolar septum with type II pneumocyte with chromatin margination (black arrow). Alveolar macrophages were also shown. (Toluidine blue x1000).

Figure 20: Transmission electron micrograph of a DEHP recovery rat alveolar tissue showing type II pneumocyte with partially empty lamellar bodies. (TEM x 12000).

Figure 21: Transmission electron micrograph of a DEHP recovery rat alveolar tissue showing alveolar macrophage with condensed heterochromatin, loss of lysosomes and loss of the short microvilli. (TEM x 15000).

into flattened type I pneumocytes, or might due to cellular proliferation. However, in this study we have noticed many mitotic figures in type II pneumocytes that could due to the effect of peroxisomal proliferation [17]. Also, chronic inflammatory process induces conversion of bone marrow mesenchymal stem cells into type II pneumocytes [23], leading to increase their number. Additionally, PPARs help in increasing translocation of triglycerides to type II pneumocytes and keep them in the surfactant secreting phenotype and preventing these cells from migration and conversion into flattened type I pneumocytes [10].

On the other hand, our electron microscopic results showed many morphological changes in type II pneumocytes in animals treated with DEHP. The changes included nuclear chromatin margination and condensation, cytoplasmic vaculation and disturbed mitochondrial cristae. These changes in type II pneumocytes are similar to the apoptotic and necrotic picture that was described in many studies [24-26] and was contributed due to the toxic effect of DEHP. Yao et al. [25] demonstrated that exposure to DEHP results in the enhanced production of Tumor Necrosis Factor-A (TNFA) and its consequent initiation of cell apoptosis through the activation of the FASL/FAS signaling pathway [25].

The disturbed lamellar bodies that we had noticed in this study might affect the surfactant production, secretion and/or readsorption by type II pneumocytes [21].Also, our result showed loss of lysosomes from macrophages, which is in agreement with others, where they have reported that DEHP-induced release of lysosomal enzymes from cultured alveolar macrophages associated with constriction and edema of pulmonary vessels [27,28].

After stopping DEHP administration for 2 weeks, the lung alveolar tissues showed mild histological and ultrastructure improvement, but complete recovery was not achieved. This was evident by the reduction of the number of type II pneumocyte, alveolar macrophages and decreased thickness of interalveolar septa in this group when compared to DEHP treated group. The absence of normal alveolar tissue architecture indicates that the DEHP induced chronic lung toxicity that seems to be duration or dose dependant [29].

Unfortunately, there was little information about the parameters determining the degree by which DEHP migrates from medical devices as temperature, surface-to-volume ratios, nature of surrounding media and its agitation, storage time, thermodynamic properties of DEHP as its vapor-pressure and degree of PVC degradation. The influence of these parameters on the leaching of DEHP is important in assessing the best condition for use of medical devices to reduce the release of the plasticizer and, as a consequence, its absorption from the patients. Other points which are unclear; the DEHP metabolizing rates across species and ages, actual toxic concentrations of DEHP and/or its metabolites, and the different administration routes in order to minimize the harmful effect of DEHP.

We can conclude that DEHP has a toxic effect on the alveolar tissues resulting in different histological changes that could persist after stopping its exposure. However, further studies are needed to determine the possibility of utilizing protective agents to overcome the toxic effects of DEHP, or stopping it for longer duration.

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