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Histological Study of the Possible Protective Effect of *Spirulina platensis* on Dibutyl Phthalate (DBP)-induced Pulmonary Alveolar Changes in Adult Male Albino Rats

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Phthalates are known to be major environmental hazards. Dibutyl phthalate (DBP), a commonly used phthalate ester, is present in a variety of products. Humans can be exposed to DBP from various sources, which can release it into biological fluids and cause various health problems by penetrating different tissues in the body. The aim of this study was to investigate the effects of DBP on pulmonary alveoli in rats and to assess the mitigating influence of S. platensis. The study involved 30 young adult male albino rats, which were divided into 3 groups (n = 10 each): control, group II (rats treated with phthalate ester (DBP; 50 mg/kg body weight/day)), and group III (Spirulina-protected animals given phthalate ester (DBP; 50 mg/kg body weight + Spirulina (200 mg/kg body weight/day)). The study revealed that alveolar tissues in the groups treated with DBP showed significant increases in collagen deposition and inflammatory cellular infiltration. Furthermore, the numbers of type-II pneumocytes and alveolar macrophages were significantly increased. However, most of these effects were ameliorated by Spirulina platensis. These findings suggest that Spirulina may have potentially beneficial effects on pulmonary alveoli by mitigating the toxic effects of DBP.

Keywords: Dibutyl phthalate (DBP), *Spirulina*, Pulmonary alveolar type-II pneumocytes, Rats.

Environmental pollution caused by chemicals resulting from human activities poses a significant threat to human and animal health, and it has been a global concern. Chemical contamination in food processing can occur through naturally occurring contaminants or as a result of interaction between food components and packaging materials during food processing and manufacturing, which can lead to the migration of some toxic compounds into food[1-5]. Although governments have taken essential steps to reduce hazardous pollutants in food, extra efforts are required to reduce the health risks and diseases associated with chemical food contamination.

Endocrine-disrupting chemicals (EDCs) are a class of synthetic compounds that are highly diverse and used in different industrial fields. Insecticides, plasticizers (phthalates), and plastics (bisphenol A) are among the most common chemicals that pose threats to human health, and

they have the potential to cause morbidity[6-12]. The use of plastics has led to environmental problems not only because they increase the amount of waste produced, but also because phthalates are found in adhesives, paints, air fresheners, and personal care products. Dibutyl phthalate (DBP) is a common phthalate ester found in various products, including food packaging, personal care products, children's toys, and medical devices. When plastics are exposed to high temperatures, hazardous components are leached out or migrate into the environment in the form of microplastics (MPs) or nanoplastics. These small forms of plastic accumulate in food chains and are accessible to human exposure via eating, skin contact, and inhalation. MPs are subdivided into two main groups: primary and secondary MPs. Primary MPs are used in the industry as plastic pellets and in personal care products such as toothpaste, nail varnish, sun creams, scrubs, and bath gels. In contrast, secondary MPs are produced from excess plastic dispersed into the environment, and they are steadily degraded due to photo- and thermo-oxidative processes and mechanical abrasion, leading to their accumulation in the human body. Textile fibers and manufacturing packings discharged into washing water from machine-washed clothes are the principal sources of these litter particles[13-19]. Epidemiology-based studies have shown that exposure to chemical contaminant has been associated with a variety of health problems including hematotoxicity, neurotoxicity, cardiotoxicity, nephrotoxicity, hepatotoxicity, and endocrine disruption[9-11]. A functional endocrine system is required for normal growth, development, and maintenance. However, in recent years, many environmental pollutants such as phthalate esters have been shown to interfere with hormonal activity. Nutritional interventions for supporting optimal immune system function are frequently overlooked in public health. Majority of micronutrients have pleiotropic effects on immunological function. Hence, *Spirulina,* as a natural "functional food", should be investigated regarding its potential to ameliorate nutritional shortages and protect the immune system[20-33].

Spirulina cyanobacteria belomgs to blue-green algae. This microorganism has been used as a source of protein and vitamins for people, without resulting in any noticeable side effects. It is already available in health food stores as a dietary supplement in a range of formulations and tablets or drinks. Moreover, it has been recognized as non-toxic in many toxicological investigations. Numerous studies on the efficacy and clinical use of *Spirulina* in management of many illnesses has suggested that this alga has antiviral, anticancer, and anti-allergic properties[34-42].

Acute respiratory tract infections are major causes of morbidity and mortality around the world. Nowadays, COVID-19 is the main cause of death locally and worldwide. Although, the mitigating influence of *Spirulina* has been examined in a variety of tissues, only few studies have investigated its benbeficial effects on pulmonary tissue. The present research was aimed at investigating the chronic toxicity of DBP in rat pulmonary alveoli, as well as the mitigating influence of *S. platensis* on the process.

MATERIALS AND METHODS

Experimental design

A total of 30 adult male albino rats weighing 150 to 200 g, were used in this study. The rats were kept in a room in an environment with equal durations of light and darkness at 24 ± 1°C, and humidity of 50 ± 10 %. The study received approval from the ethical authority of our Medical Faculty. The animals were divided into three sets, each with 10 animals. Rats in control group comprised 2 equal sub-sets given either normal feed or *S. plantensis* (0.20 g/kg bw daily)[43]. Group II received dibutyl phthalate (DBP; 50 mg/kg bw/day), while the 3rd group was given *S. platensis* (0.20 g/kg bw daily), in addition to DBP (0.050 g/kg bw daily). Each treatment was administered for 2 months via gavaging. Dibutyl phthalate (DBP) was supplied by Sigma Company, St Louis, MO, USA. The blue-green alga was obtained in powder form from a local vendor. Daily body weights, as well as feed and water consumption, were recorded. Daily treatment doses were adjusted in line with body weight changes. The doses were chosen based on the overall no-observed-adverse-effect level (NOAEL) for DBP (50 mg/kg bw/day) which was reported by other researchers[44].

Anatomical studies

At designated times, the rats were

euthanized using overdose of sodium pentobarbital (200 mg/kg). The lungs were excised and examined for any changes in shape or weight. The length of each lung was measured, from the most prominent point in the apex to a point in the middle of the basal surface, while the width was measured from the middle of the hilum to the point meeting this point horizontally on the sternocostal surface.The organs were weighed using mini scale. Then, the data were input into IBM SPSS 20.0 software. The results are presented as mean ± SD, i.e., minimum and maximum. Data with normal distribution were compared amongst multiple groups with analysis of varaince.

Tissue processing for microscopy

The right lung tissue was processed for microscopy using standard procedures. These involved tissue fixation in HCHO, clearing, dehydration, sectioning, H&E staining and routine examination under a light microscope. The other lung tissue was fixed in glutaraldehyde solution (3%), followed by processing for TEM. Electron microscopy was done, and photographs of relevant fields were obtained using Joel 100CX TEM[45].

RESULTS

In this research, DBP administration for 8 weeks did not result in death or changes in appearance in any rat group, relative to control. No apparent morphological and anatomical changes were detected during dissection of the lung in any of the experimental groups, either in the color of the organ, or in its shape. Lung weight was within the normal range of 385-395 mg (for the right lung) and 265-272 mg (for the left lung) in all groups (Table 1). The average external length of the right lung was 2.8-3.4 cm, while that of the left lung was in the range of 1.8-2.3 cm. The average values of external width for the right and right lungs were 0.5-1-0 and 0.4-0.8 cm, respectively. As shown in Table 1, there were no significant differences in these parameters in left lung in all groups (positive, negative control, treated, protected; $p > 0.05$).

Light microscopy (H&E) results

Rat lung paraffin slices from controls (-ve and +ve) had essentially normal alveolar features (Figure 1 A). Relative to controls, group II (experimental group, i.e., DPB-treated), histological sections of rat right lungs of group II revealed presence of pulmonary lesions reflected in some collapsed alveoli and distended alveoli, thickened inter-alveolar septa due to cellular infiltration as well as hyaline material, proliferation of type II pneumocytes, foamy macrophages and congested blood vessels. There were desquamated cells inside the alveolar lumen (Figures 1 B and 1 C).

In group III (protected group), co-treatment with *S. platensis* and DBP produced significant protection of alveolar morphorlogy which is evident in mitigation of alveolar lesions, except for slight enlargement of inter-alveolar septae due to infiltration of cells associated with mild congestion of blood vessels. Several bloated alveoli were lined with more or less normal type I and II pneumocytes. A slight thickening appeared in the inter-alveolar septa due to collagen fibers and few cellular infiltrations. Moreover, the numbers of macrophages and septal cells were less than those in group II (Figures 1 D and 1 E).

Data of TEM in +ve and -ve control rats revealed normal morphorlogy of alveoli with distended walls lined by type I pneumocytes and

Table 1. Lung length, width, and weight in each group

Group	Right lung			Left lung		
	Length	Width	Weight	Length	Width	Weight
Group Ia	3.07±0.23	0.77±0.19	390.45±3.30	2.02±0.17	0.60±0.14	267.65±3.15
Group Ib*	3.02±0.31	0.68±0.19	390.28±3.14	1.95±0.19	0.62±0.12	269.17±1.57
Group II	3.08±0.23	0.75±0.19	390.00±2.98	2.02±0.17	0.60±0.14	268.72±1.71
Group III	3.05±0.26	0.73±0.18	390.88±1.61	2.05±0.19	0.60±0.14	268.30±1.43
F	1.25	2.07	0.882	1.98	0.874	0.41
P	0.365	0.105	0.432	0.211	0.411	0.811

clear cytoplasm, and type II pneumocytes with apical microvilli facing alveolar lumen, indicating characteristic lamellated structure (Figure 2).

In DPB-treated rats, lung tissue showed collapsed alveoli demarcated with abnormal type II pneumocytes having empty lamellar bodies. Alveoli separation with thick interalveolar septa occurred due to infiltration by different cells: mononuclear inflammatory cells, septal cells or foamy macrophage, and desquamated cells in the lumen, in addion to significant increase in collagen deposition (Figures 3 and 4).

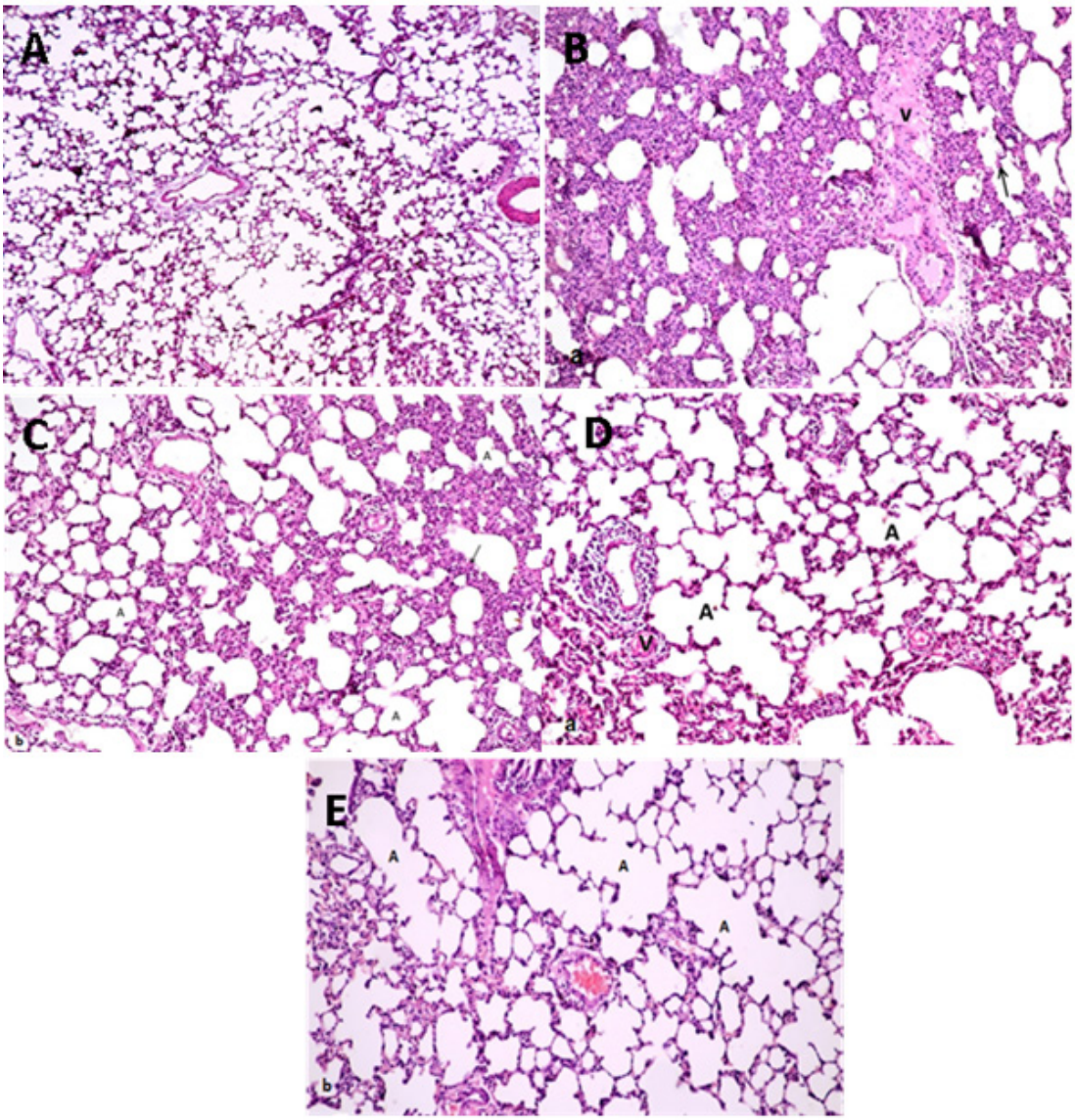

Fig. 1. (A) Photomicrograph of control rat lung (group I) showing, normal architecture of the alveoli separated by very thin interalveolar septa. The alveoli are lined by flat type I pneumocytes and rounded type II pneumocytes. B & C: Group II (DBP-treated group) showing collapsed alveoli (A) separated by very thick interalveolar septae. Note the mononuclear cellular infiltration, congestion of blood vessels (V), acidophilic hyaline material, and numerous foamy macrophages. D & E: group III (concomitant administration of DBP and spirulina), showing areas of collapsed alveoli and other distended, preserved lung architecture, except very mild increase in thickness of interalveolar septa, some acidophilic hyaline material, exfoliated cells and congested blood vessels containing acidophilic vacuolated material (V). H&E stain; x100.

Group III (protected group) had considerable degree of preservation of the alveolar architecture which was evident in reduced alveolar lesions, although slight enlargement of inter-alveolar septa due to cell invasion associated with mild congestion of blood vessels. There were numerous swollen alveoli lined with more or less normal type I and II pneumocytes. The inter-alveolar septa appeared mildly thickened due to collagen fibers and few cellular infiltrations. The numbers of macrophages and septal cells were less than that in group II (Figure 5).

DISCUSSION

The present study was aimed at elucidating the effect of chronic exposure of DBP on pulmonary alveoli, and the possible protective effect of *Spirulina*. The DBP treatment exacerbated alveolar injury. This may be attributed to oxidative stress which was morphologically reflected in proliferation of type II pneumocytes and alveolar macrophages, collapsed alveoli, cellular infiltrations, congestion of blood vessels and desquamated cells. It is generally agreed that nutrient deficiency may lead to changes in immunity which are demonstrated as changes in levels of T-cells, IgA antibody response, cytokines and NK cells[46-49].

Spirulina might modulate the immune system by mitigating nutritional deficiencies. Previous research have demonstrated that phycocyanin (PC), a protein extracted from cyanobacteria, has a variety of benefits such as antioxidant and anti-inflammatory properties[50-53]. Moreover, PC has been shown to reduce paraquat-induced lung damage in rats. In addition, in lipopolysaccharide-stimulated macrophages, PC, a selective cyclooxygenase-2 inhibitor, promotes apoptosis.These findings corroborate the findings in this study which showed reductions in macrophages and desquamated cells when *Spirulina* and DBP were given together. Furthermore, other studies revealed that *Spirulina* inhibited the release of histamine from mast cells. Indeed, analysis of blood samples of volunteers before and after

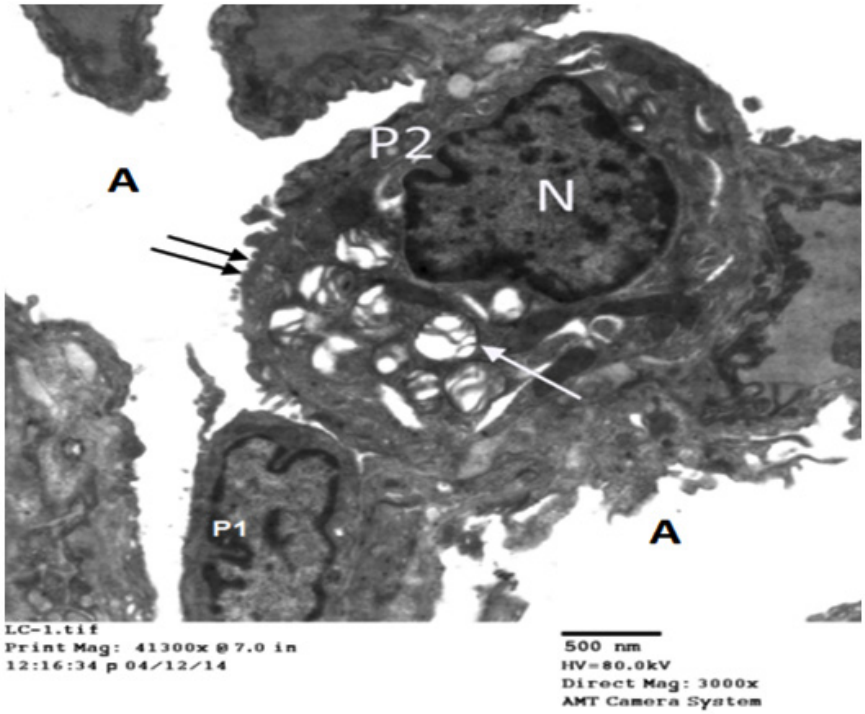

Fig. 2. Electron micrograph of group I revealing open alveoli (A) bordered by flat nuclei of type I pneumocytes (P1) and type II pneumocytes (P2) with characteristic lamellated structure (‘!) and apical microvilli (mv).

oral administration of *Spirulina platensis* has shown that it has anti-inflammatory and antiviral properties[42,43]. orrespondingly, in managing patients with allergic rhinitis, *Spirulina* administration resulted in considerably ameliorated physical manifestations and symptoms such as nasal congestion, nasal discharge, sneezing, and itching[54-63]. Phthalate-mediated pulmonary lesions may precipitate fibrotic changes. Phthalates produce these delecterious changes in epithelial cells through complex molecular mechanisms mediated by oxidative insults which ultimately result in apoptotic lesions, generation of pro-fibrosis cytokines, and massive deposits of extracellular matrix. The end results of these processes is the establishment of pulmonary fibrosis. These are in agreement with the results obtained in this study, as is evident in presence of desquamated and exfoliated cells in the lumen of the alveoli, as well as increases in collagen fibers. Other study proposed that autophagy is an upstream event triggering lysosomal membrane permeabilization that would lead to apoptosis through the lysosomal-mitochondria pathway as described by others[40]. On the other hand, *in vitro*, an aqueous extract of *Spirulina* reduced HIV-1 multiplication in human T-cells, peripheral blood mononuclear cells, and Langerhans cells. Consequently, employing

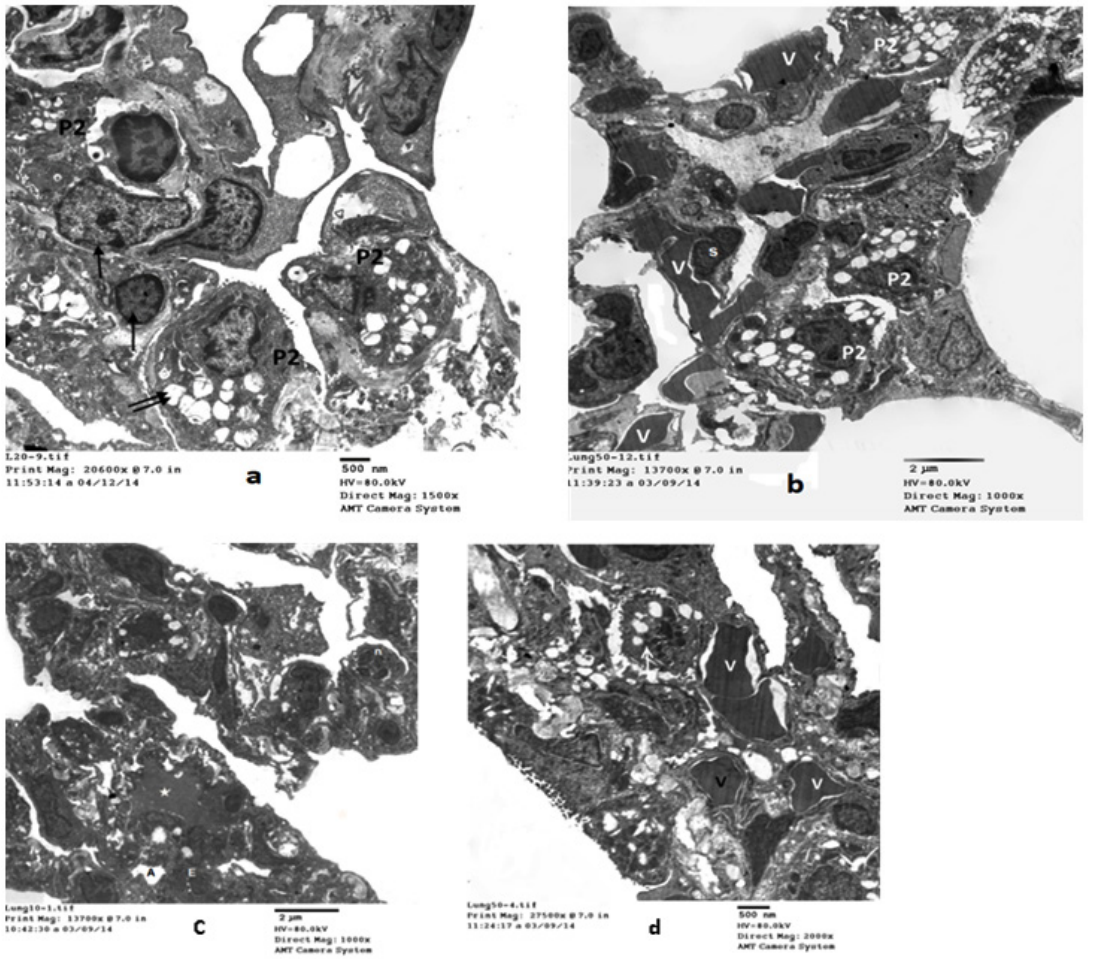

Fig. 3. Electron microscopy showing DBP-treated rat lung. a & b: show alveolar lesions. Collapsed alveoli are bordered by evidently vacuolated pneumocyte type II (P2) with empty lamellated structure, numerous activated macrophages with vacuolated cytoplasm (foam cells) and congestion of blood vessels (V). c & d: show thick interalveolar septa due to mononuclear cellular infiltration (‘!) and congestion of the blood vessels (V). Note type II pneumocytes filled with empty lamellated structure (P2).

herbs and algae products with proven antiviral capabilities in combating specific viruses could be done through immunomodulation, even after the illness has been established[63-65]. These findings are consistent with those obtained in this study, as evidenced in reduction in cellular infiltration when *Spirulina* and DBP were given together. Of course, before any conclusions can be formed, these potential results must be further investigated in animal models and humans.

It has been suggested by many researchers that the antioxidant and immuno-modulatory properties of *Spirulina* may have a tumor-destructive implication, thereby making it play a role in cancer prevention[64-66]. The anti-tumor potential of *Spirulina* are thought to be derived from â-carotene, a potent antioxidant. Nevertheless, the link between carotene contents and cancer cannot be proved because the pathogeneisis of cancer is typically complex. Currently, many studies have demonstrated the safety of *Spirulina* supplements, with few adverse effects, but its full potential as a medication is unknown.

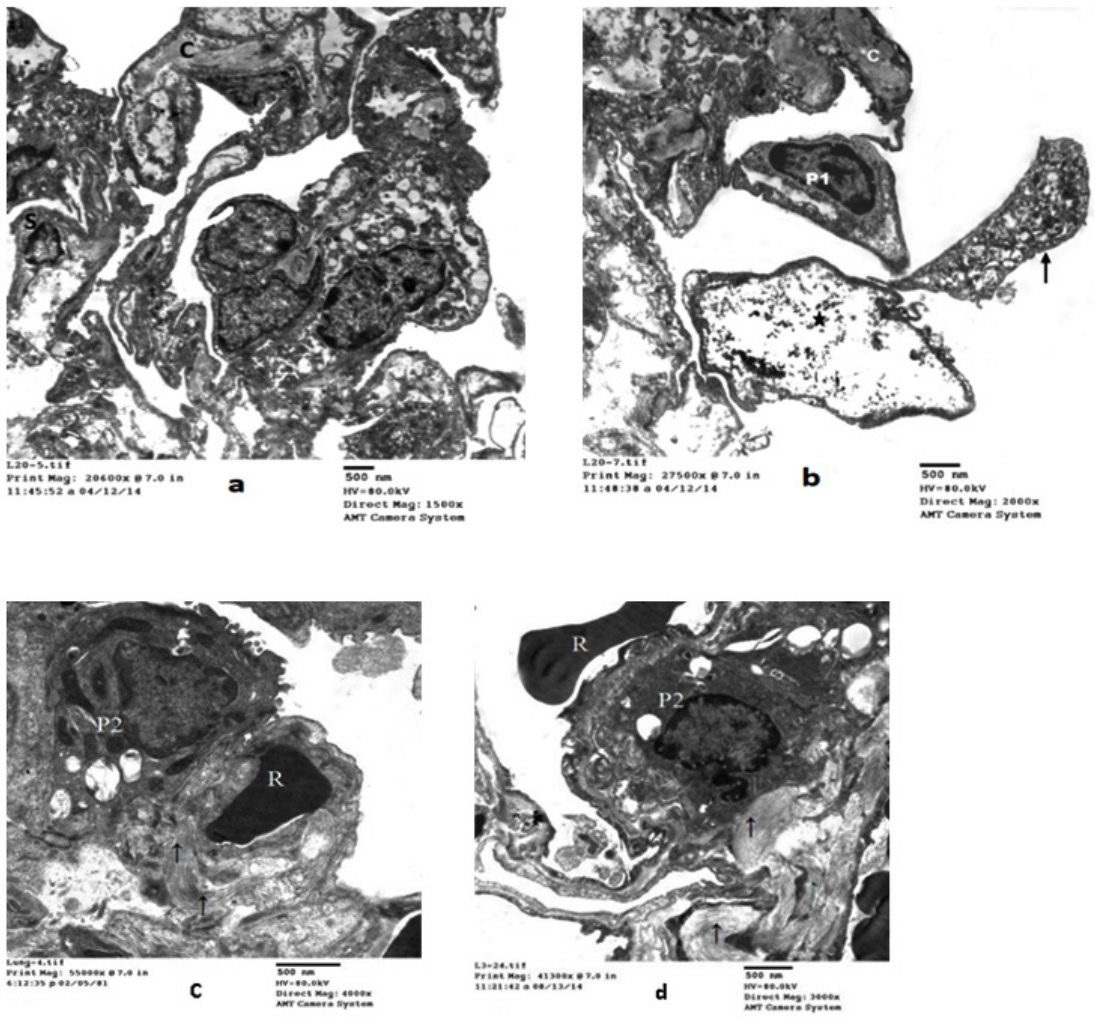

Fig. 4. Electron micrographs of rat lung in group II (DBP-treated rats). a & b: show thick interalveolar septa due to mononuclear cellular infiltration. Note type II pneumocytes filled with empty lamellated structure (P2), and cellular debris and desquamated cells in the lumen of the alveoli (‘!). c & d: show thick interalveolar septae due to mononuclear cellular infiltration (‘!) and congestion of the blood vessels. Note type II pneumocytes filled with empty lamellated structure (P2) and a significant increase in collagen deposition (‘!).

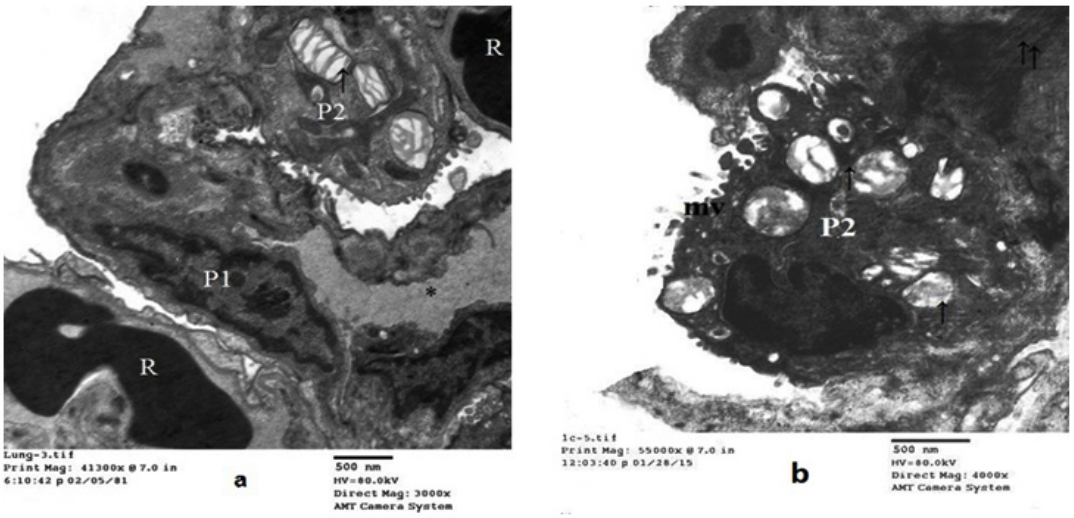

Fig. 5 a & b. Electron micrograph of group III rat lung (protected group) revealed considerable degree of preservation of the alveolar architecture evident in decreased alveolar alterations, apart from low degree of swelling of inter-alveolar septae by cell influx associated with mild congestion of blood vessels. Several alveoli were swollen and bordered with more or less normal types I and II pneumocytes. The inter-alveolar septae appeared mildly thickened due to collagen fibers and few cellular infiltrations. The numbers of macrophages and septal cells were less than that in group II.

CONCLUSION

Phthalates have a large scale of applications, and their usage is an extensive source of environmental pollution. Exposure to phthalates has been linked to poor pulmonary health, including lung fibrosis. *Spirulina* has a potential beneficial effect in mitigating the toxic effects of phthalates on the pulmonary alveoli. Thus, it is critical to educate the public on the dangers of phthalates so as to enhance their well-being. Therefore, the public must recognize the sources and pathways of phthalate exposure so that they significantly avoid them, particularly in those liable to exposure.

Ethical registration

The animal study protocol was approved by the Institutional Ethics Committee Faculty of Medicine, University of Mutah (protocol code T342 and 30.08.2021).

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